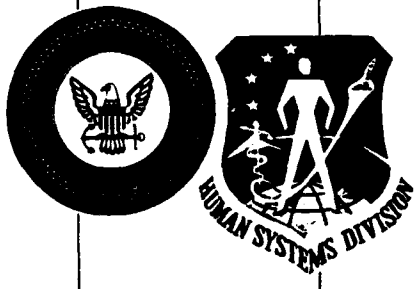

Review of DOD Malaria Research Programs

Joint Technology Coordinating Group 2
Infectious Disease

Bethesda, Maryland
20-21 May 1992

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DoD Malaria Research Review

Malaria is a present and historic threat to deployed United States military forces. Presently, prophylactic drugs, insect repellents and insecticides are the only means of prevention. Malaria parasites are capable of developing resistance to new drugs in a few years. Insect control measures are never more than temporary and incompletely effective measures. A major deterrent to optimal use of antimalarial drugs and insect control measures is their huge expense. There is a widely accepted need for prevention of malaria by immunization.

For over 25 years, the Department of Defense (DoD) has invested in malaria vaccine and drug development by U.S. Army and Navy investigators in this country and overseas. The DoD is the world leader in both approaches to malaria prevention. A number of new antimalarial drugs, which are in general use, and several emerging possibilities for vaccines have resulted from this work. In Fiscal Year 1992, the United States Army Medical Research and Development Command is investing $9.7 million, or 19.1% of the entire Infectious Disease Budget, on malaria vaccine and drug development. This review is intended to assess the progress made and the appropriate level of investment.

The principal laboratory managers of the malaria research programs have been charged to present an assessment of the risk of malaria to military operations and to identify their research objectives, scientific approaches, technological barriers, and emerging products for contingency use if required in the next year. The emphasis is on the science conducted, not program administration. Time does not permit inclusion of research on insect vectors.

Three outside experts have been invited to review the material. They have been asked to comment on program progress, unnecessary duplication of effort, research deficiencies, interaction between laboratories, and available resources.

This review is sponsored by the Joint Technology Coordinating Group-2 for Infectious Disease, a tri-service subcommittee under the Armed Services Biomedical Research Evaluation and Management Committee. During the past two years, the JTCG-2 has endorsed malaria vaccine development and anti-parasitic drug development as equally important areas of emphasis within the Infectious Disease Research Program. This review should serve as a milestone for measuring future progress in malaria research.

WILLIAM H. BANCROFT
Colonel, Medical Corps
Chairman
Joint Technology Working Group-2

DOD Malaria Research Review

May 20-21, 1992

Holiday Inn Bethesda
Bethesda, MD

AGENDA

Malaria Vaccine Program

Wednesday, 20 May 1992 ***NMRDC/Morning Session***

0800	Welcome	*COL Bancroft*
0805	The Past, Current, and Potential Impact of Malaria on Navy and Marine Personnel: The Requirement for Vaccines	*CAPT F. Stephen Wignall* *Navy Environmental and Preventive Medicine Unit No. 6*
0820	Malaria Vaccine Program, NMRDC	*CAPT (sel) Stephen L. Hoffman* *Malaria Program* *Naval Medical Research Institute*
1000	**Break**	
1015	Malaria Vaccine Program, NMRDC (cont'd)	*CAPT (sel) Stephen L. Hoffman* *Malaria Program* *Naval Medical Research Institute*
1115	Discussion	
1200	**Lunch**	

WRAIR/Afternoon Session

1300	Malaria as a Disease Threat for Military Personnel	*MAJ Bruno Petrucelli* *Department of Advanced Preventive Medicine* *WRAIR*
1310	Program Overview	*LTC W. Ripley Ballou* *Department of Immunology* *WRAIR*

Malaria Vaccine Program (cont'd)

Wednesday, 20 May 1992 — *WRAIR/Afternoon Session (Continued)*

Technical Presentations

1355	Selected Topics in Basic Research	*LTC W. Ripley Ballou* *Department of Immunology* *WRAIR*
1400	▪ EBA-175: An Asexual Stage Antigen Involved With Parasite Invasion of Red Blood Cells	*COL David Haynes* *Department of Immunology* *WRAIR*
1410	▪ Characterization of Cytoadherence Antigens	*CPT(P) Francis Klotz* *Department of Immunology* *WRAIR*
1420	▪ Discussion	
1425	Exploratory Development of Malaria Vaccines	*LTC W. Ripley Ballou* *Department of Immunology* *WRAIR*
1430	▪ Engineered Attenuated Salmonella Vector Malaria Vaccines	*Dr. Richard Warren* *Department of Bacterial Diseases* *WRAIR*
1440	▪ Engineered Vaccinia and BCG Vector Malaria Vaccines	*MAJ D. Gray Heppner* *Department of Immunology* *WRAIR*
1450	▪ Development of Methods To Induce and Measure CMI	*Dr. Urszula Krzych* *Department of Immunology* *WRAIR*
1500	▪ Discussion	
1510	**Break**	

Malaria Vaccine Program (cont'd)

Wednesday, 20 May 1992 — *WRAIR/Afternoon Session (Continued)*

1530	Overview of DOD Malaria Vaccine Trials	*LTC W. Ripley Ballou* *Department of Immunology* *WRAIR*
1545	▪ Case Study: R32ToxA Phase I-IIb	*LTC Daniel Gordon* *Department of Immunology* *WRAIR*
1555	▪ Analysis of R32ToxA Phase IIb Data	*COL Jerald Sadoff* *Division of Communicable Diseases and Immunology* *WRAIR*
1605	▪ Discussion	
1610	Enhancement of Malaria Vaccine Production Capabilities	*LTC W. Ripley Ballou* *Department of Immunology* *WRAIR*
1615	▪ Establishment of In-House Fermentation Capabilities	*Dr. Luther Lindler* *Department of Immunology* *WRAIR*
1625	▪ Establishment of a cGMP Peptide Synthesis Facility	*Dr. Carolyn Deal* *Department of Bacterial Diseases* *WRAIR*
1635	▪ Discussion	
1640	Program Requirements for FY93—Budget and Personnel	*LTC W. Ripley Ballou* *Department of Immunology* *WRAIR*
1650	Discussion and Comments From RAD-1 and Extramural Panel	

Experimental Therapeutics

Thursday, 21 May 1992 — *WRAIR/Morning Session*

0800	Introduction and Program Overview	*COL Brian G. Schuster* *Division of Experimental Therapeutics* *WRAIR*
0820	Drug-Resistant Malaria in Indochina	*CPT Colin K. Ohrt* *Department of Medicine* *AFRIMS*
0830	Basic Research in Drug Discovery and Design	*LTC Wilbur K. Milhous* *Division of Experimental Therapeutics* *WRAIR*
	▪ Molecular Biology and Clinical Relevance of Multiple Drug Resistance	
0840	▪ Isolation and Characterization of Transport Proteins in Malaria and Leishmania Which Mediate Drug Efflux	*MAJ Max Grogl* *Division of Experimental Therapeutics* *WRAIR*
0855	▪ New Biochemical Targets	*Dr. Malcolm J. Gardner* *Division of Experimental Therapeutics* *WRAIR*
0910	▪ Applications of Molecular Modeling to Drug Discovery and Design	*Dr. Jean M. Karle* *Division of Experimental Therapeutics* *WRAIR*
0925	▪ Traditional Medicine and Natural Products in Drug Discovery and Design	*Dr. Marice M. Iwu* *Division of Experimental Therapeutics* *WRAIR*
0940	**Break**	
1000	The Importance of Reliable and Reproducible Laboratory and Animal Models	*LTC Wilbur K. Milhous* *Division of Experimental Therapeutics* *WRAIR*
	The Development of Resistance Modulators as Candidate Drugs	
1015	▪ WR268954 as a Resistance Modulator	*Dr. Dibyendu De* *Division of Experimental Therapeutics* *WRAIR*

Experimental Therapeutics (cont'd)

Thursday, 21 May 1992 — *WRAIR/Morning Session (Continued)*

	New Leads	
	▪ Artemisinin Analogues	
1030	- In-House and Extramural Synthetic Efforts	*Dr. Robert R. Engle* *Division of Experimental Therapeutics* *WRAIR*
1045	- Evaluation of Artelinic Acid and Analogues	*Dr. Daniel L. Klayman* *Division of Experimental Therapeutics* *WRAIR*
1055	- Preclinical Studies of Artemisinin Derivatives and Metabolites	*COL Thomas G. Brewer* *Division of Experimental Therapeutics* *WRAIR*
1110	▪ Synthetic Peroxides (Totroxanes and Trioxanes) and Bis-Quinolines	*MAJ Steven Andersen* *Division of Experimental Therapeutics* *WRAIR*
1120	▪ Floxacrine Analogues, Antibiotics, Folate Antagonists, 8-Aminoquinolines, and Guanylhydrazones	*LTC Wilbur K. Milhous* *Division of Experimental Therapeutics* *WRAIR*
	Phase I and II Clinical Pharmacology	
1130	▪ WR238605	*MAJ Ralf P. Brueckner* *Division of Experimental Therapeutics* *WRAIR*
1140	▪ Halofantrine	*COL Brian G. Schuster* *Division of Experimental Therapeutics* *WRAIR*
1145	Summary and Strategies for Future Directions in Basic Research in Antimalarial Drugs	*LTC Wilbur K. Milhous* *Division of Experimental Therapeutics* *WRAIR*
1150	Summary of Problems and Critical Issues in Malaria Drug Development	*COL Brian G. Schuster* *Division of Experimental Therapeutics* *WRAIR*
1200	**Lunch**	

Executive Session

Thursday, 21 May 1992 — *Afternoon Session*

1300	Program Overview	*COL Bancroft*
1330	Discussion	*Advisors and Program Managers*

- Vaccine Development
- Drug Development

1600	Recommendations	
1700	Adjournment	

Information Paper.1

Malaria Program
Naval Medical Research Institute
CDR Stephen L. Hoffman

20 May 1992

SUBJECT: Malaria Vaccine Development

OBJECTIVE: To protect against malaria by actively or passively inducing antibodies that prevent sporozoites from effectively invading hepatocytes.

STRATEGY:

ACTIVE IMMUNITY

1. Identify targets on the sporozoite surface accessible to antibodies
   a. Repeat region of the CS protein
   b. Flanking regions of the CS protein
   c. Sporozoite surface protein 2 (SSP2)
   d. Other?
2. Develop methods for determining that antibodies are protective.
3. Develop methods for consistently producing high levels of protective antibodies working in mouse model systems, non human primate models, and in humans.
   a. Targets (B epitopes)
   b. Carrier proteins (T helper epitopes)
   c. Delivery systems (adjuvants, vaccine construction, liposomes, etc.)
4. Test such vaccines for safety, immunogenicity, and protective efficacy in humans exposed to experimental challenge.
5. Test such vaccines for safety, immunogenicity, and protective efficacy in small and then large field studies.
6. Field the vaccines.
7. Determine parasite molecules involved in the recognition, attachment, and invasion of sporozoites to hepatoctyes, and induce antibodies that interfere with this interaction.

PASSIVE IMMUNITY

1. Produce human monoclonal antibodies against sporozoites that can be used to protect humans by passive transfer.
2. Test such reagents for safetey, immunogenicity, and protective efficacy in non-human primates and in humans.
3. Identify the target of these protective monoclonal antibodies and formulate vaccines for active immunization that induce polyclonal antibodies of the same specificity.

CURRENT STATUS:

ACTIVE IMMUNITY

1. The repeat region of the CS protein was identified as a target for vaccine development because Mabs against this region of the *P. berghei*, *P. yoelii*, and *P. vivax* CS proteins protect against malaria when given in passive transfer, and because monoclonal and polyclonal antibodies

against the *P. falciparum* CS protein block sporozoite invasion and development within hepatocytes. Vaccines designed to produce such antibodies in the *P. yoelii* rodent malaria and *P. vivax* Saimiri model systems, and in the human *P. falciparum* and *P. vivax* systems have been constructed and tested. In the *P. yoelii* rodent and *P. vivax* monkey systems none of the vaccines gave protection. In the P. falciparum human systems 15-20% of volunteers are protected, and another 15-20% have a delay in patency indicating >95% reduction in sporozoites effectively invading hepatocytes. Numerous combinations of B and T epitopes and delivery systems have been tested in mice and in humans for safety, immunogencity, and protective efficacy. Numerous assays have been tested for their capacity to predict protective immunity. In the rodent system it has been shown that antibodies of all subclasses, and antibodies induced by immunization with synthetic peptides can protect, and that Mab-mediated protection is predicted by antibody affinity assays, and that fine specificity of antibodies may be critical for protection. Such studies are just beginning for polyclonal vaccine-induced antibodies.In humans, none of the assays have a high positive predictive value for predicting protection.

2. Antibodies against PfSSP2 have been shown to block sporozoite invasion and development of P. falciparum sporozoites into human hepatocytes. In the P. yoelii system it has been shown that antibodies to PyCSP seem to upregulate the expression of PySSP2. This raises interesting questions regarding synergistic activity of two antibodies.
3. Identification of parasite and hepatocyte molecules involved in the recognition, attachment, and invasion of sporozoites into hepatocytes is underway. Sulfated glycolipids have been shown to be involved in the recognition of CS protein and sporozoites for hepatocytes.

PASSIVE IMMUNITY

1. Humanized mouse Mab: A mouse Mab, NFS2 (Navy falciparum sporozoite 2) that blocks sporozoite invasion and development in human hepatoctyes >98% has been "humanized" using 2 methods. First by cloning the gene encoding the variable region of this Mab into vectors that include the heavy and light chain constant regions of human IgG. Second by sequencing the gene encoding the variable region and cloning oligonucleotides based on the CDRs into a human IgG backbone. Both have been expressed in COS cells. These "humanized" Mabs recognize the repeat region of the CS protein in ELISA and sporozoites by IFAT with the same intensity on a μg/ml basis as does NFS2. Preliminary studies; they both block sporozoite invasion into hepatocytes in vitro.
2. Combinatorial libraries (human Fab): A combinatorial library from B cells from a volunteer immunized with radiation attenuated P. falciparum sporozoites has been produced. Screening of the library is beginning this week.
3. Human Mab: PBL from a volunteer immunized with irradiated *P. falciparum* sporozoites have transplanted into scid mice and will be used to produce human Mabs.

TECHNOLOGICAL BARRIERS:

1. Sporozoites cannot be grown in vitro.
2. *P. falciparum* sporozoites only invade and develop within human hepatocytes, and some non human primate hepatoctyes.
3. Optimal formulations of target, carrier protein, and vaccine delivery system have not been defined.
4. Models for *P. falciparum* sporozoite infection of monkeys are expensive and not well substantiated.
5. Production and purification of recombinant and synthetic peptides and other vaccine delivery systems is expensive, difficult, and slow.

PLANS:

AC TIVE IMMUNIZATION

1. Human studies: We will continue to test vaccines designed to produce protective antibodies against the CS protein, in an attempt to improve the three components of the vaccine (B epitopes, T epitopes, and delivery system). These will include synthetic peptides (branched chain polymers, straight chain polymers, constrained peptides), recombinant proteins delivered with newly defined carrier proteins and delivery systems, and live vectored vaccines. These vaccines will be combined with vaccines designed to produce immune responses including antibodies against other regions of the CS protein and against other malaria proteins, especially MSP-1.
2. Animal models: Work will continue in the 6.1 and 6.2 areas to refine our vaccine formulations so as to produce polyclonal antibodies with the protective efficacy of monoclonal antibodies.

PASSIVE IMMUNIZATION

1. Chimeric and humanized NFS2 will undergo pre-clinical testing, and after IND submission, studies of safety, immunogenicity, and protective efficacy in humans.
2. The first priority with the combinatorial libraries will be to produce Mabs against the CS protein. However, in the fall of 92 work will begin to produce libraries from spleens of trauma victims from malaria endemic areas so as to produce Fabs against blood stage proteins.
3. Work producing human Mabs in scid mice will continue.

RESOURCE REQUIREMENTS:

1. *P. yoelii, P. falciparum, P. vivax,* and *P. berghei* sporozoites.
2. Human hepatocytes.
3. Production of GMP grade synthetic peptides and recombinant proteins.
4. Large numbers of volunteers in the United States.
5. Study sites in malaria endemic areas.
6. Continued immunization of humans with irradiated P. falciparum and P. vivax sporozoites.
7. Non human primates for active and passive studies (Saimiri for P. vivax and Aotus for P. falciparum.
8. Additional laboratory space.
9. Two clinicians to conduct clinical trials.
10. Increased funding (see attached funding document).

Information Paper.2

Malaria Program
Naval Medical Research Institute
CDR Stephen L. Hoffman

20 May 1992

SUBJECT: Malaria Vaccine Development

OBJECTIVE: To protect against malaria by inducing immune responses that eliminate malaria infected hepatocytes or kill the parasite within the hepatocyte.

STRATEGY:

1. Identify the targets and mechanisms of protective immunity against infected hepatocytes provided by the irradiated sporozoite vaccine. Work in the mouse model system and then extrapolate to human malarias. Study naturally acquired immune responses to get some idea of the validity of the murine results.
2. Develop methods for duplicating this immunity by immunization with synthetic or recombinant vaccines. Work simultaneously in the mouse model system and with human malarias.
3. Identify targets and mechanisms of protective immunity not invoked by the irradiated sporozoite vaccine and develop methods for inducing such immunity.

CURRENT STATUS: Mice immunized with irradiated sporozoites have now been shown to produce CTL against the CS protein and SSP2, and humans immunized with irradiated sporozoites have been shown to produce CTL against the CS protein and T cell proliferative responses against SSP2. Methods have been developed using transfected mammalian cells as vaccines to induce CD8+ T cell-dependent protective immunity in the rodent malaria system. Work is underway to develop recombinant vaccinia, Salmonella, BCG, and Leishmania sp., soluble recombinant protein, and synthetic peptide vaccines that duplicate this immunity in the mouse model systems. The gene encoding the P. falciparum SSP2 has been characterized. Work is underway to produce human vaccines (vaccinia, Salmonella, BCG, synthetic petides, recombinant protein) that induce similar immune responses in humans. An S. typhi carrying the gene for the CS protein has been produced and we will soon test it for its capacity to induce CTL in humans.

An additional target of protective immune responses has been identified (LISA-3/Exp-1). Antibodies against the P. yoelii LISA-3 eliminate infected hepatocytes from in vitro culture. Work is proceeding with these three antigens to not only produce protective CD8+ CTL, but also CD4+ CTL, protective antibodies, and to develop methods for producing protective cytokines.

Work is actively underway to produce a new generation of vaccines that produce better CTL by targeting the protein of interest for cytosolic degradation.

TECHNOLOGICAL BARRIERS:

1. Sporozoites cannot be grown in vitro.
2. *P. falciparum* sporozoites only invade and develop within human hepatocytes, and some non human primate hepatoctyes.
3. A source of material to identify additional liver stage targets is therefore extremely difficult to come by.
3. Optimal formulations of vaccines to produce CTL and other CMI responses against defined epitopes have not been developed.
4. There is no optimal model for *P. falciparum* sporozoite infection of animals.
5. Production and purification of recombinant and synthetic peptides and other vaccine delivery systems is expensive and difficult.
6. Industrial partners have difficulty sustaining the effort required to carry out this type of vaccine development.

PLANS: These are identified in the abstracts. In short the plan is to work simultaneously in the rodent and human systems to produce vaccines that protect by attacking the infected hepatocyte. The major emphasis will be on synthetic peptides and recombinant live vectors designed to produce protective CTL against the CS protein, and SSP2, and protective antibodies that recognize LISA-3/Exp-1. There will be considerable attention to further defining the activity of antibodies and ADCC to LISA-3/Exp-1 against infected heaptocytes. In addition we will evaluated whether CTL against LISA-3 and PyMSP-1 eliminate infected hepatocytes from culture and protect against malaria in vivo. As more data is forthcoming there will be efforts to produce CD4+ CTL, and protective cytokine responses against these proteins, and against MSP-1 in hepatocytes. The first of such vaccines are now entering human trials, and we anticipate large numbers of such vaccines going into human testing in the next year or two. We will continue to refine our techniques and define the mechanisms and targets in the mouse model system, while proceeding as rapidly as possible into human studies, both in the United States and in malarious areas.

RESOURCE REQUIREMENTS:

1. *P. yoelii, P. falciparum, P. vivax,* and *P. berghei* sporozoites.
2. Human hepatocytes.
3. Production of GMP grade synthetic peptides and recombinant proteins, and live vector vaccines.
4. Large numbers of volunteers in the United States.
5. Study sites in malaria endemic areas.
6. See attached funding document for data and requirements.
7. A molecular biologist and a protein chemist are required for increasing the effort to construct vaccines.
8. An additional laboratory is required for the vaccine development and molecular immunology studies.

Information Paper.3

Malaria Program
Naval Medical Research Institute
CDR Stephen L. Hoffman

20 May 1992

SUBJECT: Malaria Vaccine Development

OBJECTIVE: To protect against malaria by inducing immune responses that prevent malaria merozoites from invading erythrocytes and/or by inducing cytokines that kill the parasite within erythrocytes.

STRATEGY:

1. Identify the B cell epitopes on PyMSP-1 recognized by the protective Mab 302 and construct vaccines (Baculovirus expressed protein, recombinant vaccinia, recombinant Salmonella typhimurium, synthetic peptides (constrained and linear peptides, and polymers) designed to induce antibodies of similar specificity. Test the animals for protection by sporozoite challenge.
2. Evaluate the effect of antibodies to LISA-3 on blood stages of P. yoelii, and antibodies to Exp-1 on P. falciparum blood stages.

CURRENT STATUS:

1. A monoclonal antibody to PyMSP-1 has been shown to dramatically reduce the level of parasitemia after challenge with P. yoelii sporozoites. When this Mab is administered with a non-protective quantity of antibodies to the P. yoelii CS protein repeat region, the combination has been shown to protect 90% of animals, providing the first example of additive or synergistic protective activity of responses against the blood and sporozoite stages of the parasite. Work is underway to duplicate this reactivity with vaccine induced antibodies.

TECHNOLOGICAL BARRIERS:

1. Sporozoites cannot be grown in vitro.
2. *P. falciparum* sporozoites only invade and develop within human hepatocytes, and some non human primate hepatoctyes.
3. Optimal formulations of vaccines to produce CTL and other CMI responses against defined epitopes have not been developed.
4. There is no optimal model for *P. falciparum* sporozoite infection of animals.
5. Production and purification of recombinant and synthetic peptides and other vaccine delivery systems is expensive and difficult.
6. Industrial partners have difficulty sustaining the effort required to carry out this type of vaccine development.

PLANS:

1. PyMSP-1/PfMSP-1. The plan is to refine the induction of protective immunity in the P. yoelii system and then proceed to the P. falciparum and P. vivax systems for induction of comparable immunity. Synthetic peptide (linear

and constrained), baculovirus expressed recombinant protein, and live vector recombinants will be produced in both the P. yoelii , P. falciparum, and P. vivax system and evaluated for their capacities to produce protective antibodies.

2. LISA-3/Exp-1. Work will proceed to determine if the antibodies with activity against infected hepatoctyes also have activity against merozoites or infected erythrocytes.

3. As outlined in information paper.1 above for attacking sporozoites, we will produce human monoclonal antibodies against PfMSP-1, Pf-EBA 175, and PfEXP-1 that can be used for passive protection.

RESOURCE REQUIREMENTS:

1. *P. yoelii, P. falciparum, P. vivax,* and *P. berghei* sporozoites.
2. Human hepatocytes.
3. Production of GMP grade synthetic peptides and recombinant proteins, and live vector vaccines.
4. Large numbers of volunteers in the United States.
5. Study sites in malaria endemic areas.
6. See attached funding document for data and requirements.

Information Paper.4

Malaria Program
Naval Medical Research Institute
CDR Stephen L. Hoffman

20 May 1992

SUBJECT: Malaria Vaccine Development

OBJECTIVE: To protect against malaria by inducing immune responses that attack the sporozoite in circulation, attack the infected hepatocyte, prevent malaria merozoites from invading erythrocytes and kill the parasite within erythrocytes.

STRATEGY:

1. This is the culmination of the other approaches and we believe will lead to fulfillment of the objectives of the Malaria Program to protect the operating forces against malaria.
2. The strategy will be to provide human monoclonal antibodies against the sporozoite, liver, and blood stage of the parasite for passive immunization, and multivalent vaccines that attack these same targets.
3. Working again in the mouse model, non-human primate model, and human systems we will construct synthetic peptide, recombinant protein, and live vector vaccines that induce immune responses against multiple targets. It will be critical to evaluate how these immune responses enhance and suppress each other.
4. We will focus on CS protein, SSP2, Lisa-3/Exp-1, and MSP-1 and will work in the P. yoelii mouse model system, the non-human primate system for P. falciparum and P. vivax, and in humans for P. falciparum and P. vivax.

CURRENT STATUS:

1. We have accomplished the first successful, reproducible **complete** protection against malaria by immunization with a subunit vaccine by immunizing with a combination of PyCSP and PySSP2 (Khusmith et al., Science, 1991). This is accomplished by immunizing with transfected mammalian cells.
2. The gene encoding PyMSP-1 has been transfected into the same system, and the gene encoding LISA-3 is being transfected.
3. We have entered into a collaboration with Virogenetics to put the same four genes into vaccinia.
4. We have begun construction of Salmonella and Leishmania constructs with the same four genes.
5. Synthetic and recombinant peptides are being produced to reflect known B and T cell epitopes on these proteins for studies of combination vaccines.
6. Analogous P. falciparum constructs are being produced.

TECHNOLOGICAL BARRIERS:

1. Sporozoites cannot be grown in vitro.
2. *P. falciparum* sporozoites only invade and develop within human hepatocytes, and some non human primate hepatoctyes.
3. Optimal formulations of vaccines to produce CTL and other CMI responses against defined epitopes have not been developed.
4. There is no optimal model for *P. falciparum* sporozoite infection of animals.
5. Production and purification of recombinant and synthetic peptides and other vaccine delivery systems is expensive and difficult.
6. Industrial partners have difficulty sustaining the effort required to carry out this type of vaccine development.
7. Essentially nothing is known about the interactions expected from multiple immune responses.

PLANS:

ACTIVE IMMUNIZATION

1. Extensive studies and trials will be carried out to develop optimal formulations of these multivalent vaccines. The system will be refined in the mouse model and non-human primate systems and validated in humans immunized and challenged in the U.S. and naturally exposed to malaria abroad.

PASSIVE IMMUNIZATION

1. Human monoclonal antibodies against PfCSP, PfSSP2, PfExp-1, PfMSP-1, and PfEBA-175 will be produced and used as cocktails to provide passive protection against malaria.

RESOURCE REQUIREMENTS:

1. *P. yoelii, P. falciparum, P. vivax,* and *P. berghei* sporozoites.
2. Human hepatocytes.
3. Production of GMP grade synthetic peptides and recombinant proteins, and live vector vaccines, and human monoclonal antibodies
4. Large numbers of volunteers in the United States.
5. Study sites in malaria endemic areas.
6. See attached funding document for data and requirements.
7. An additional molecular biologist, protein chemist, cellular immunologist, and two clinicians.
8. Two additional laboratories for vaccine production and cellular immunology studies are required.

Project Title: The history of malaria in the United States Naval Forces at war--from World War I to the Vietnam Conflict
Funding: 6.3
Principal Investigator: Christine Beadle, LCDR, MC, USN
Co-Investigator: Stephen L. Hoffman, CDR, MC, USNR

Objective: To describe the problems the naval services (particularly the ground forces) have had with malaria during this century's major campaigns.

Methods: We calculated the malarial incidence rates and noneffective ratios for the entire Navy and Marine Corps using information found in the Navy's Annual Surgeon General's reports for the time periods of interest. When available, we compared more specific unit information to these generic rates.

Progress: We described the impact of malaria on the naval forces during the World Wars, the Korean War, and the Vietnam Conflict. In all of the campaigns after World War I, we were able to demonstrate how much more severe the malarial problem was for the ground troops deployed to malarious, tropical areas than for the worldwide naval forces. Some of the conditions which worsened the malarial situation during each campaign are highlighted.

Plans: A manuscript is being prepared for journal submission (abstract attached).

Abstracts:
1. Beadle C,Hoffman SL. The History of Malaria in the United States Naval Forces at War--From World War I to the Vietnam Conflict.33RD Navy Occupational Health and Preventive Medicine Workshop, Virginia Beach, VA, Mar 92.

Publications:
1. Berg SW, Beadle C, Trump DH, eds. Navy Medical Department Guide to Malaria Prevention and Control. Navy Environmental Health Center Technical Manual 92, 3-6, 103, 1991.

Project Title: Identification and characterization of vaccine study sites.
Funding: 6.3
Principal Investigator: Trevor R. Jones, LCDR, MSC, USN
Co-Investigators: Thomas Richie, LCDR, MC, USNR, NAMRU-2
J. Kevin Baird, LCDR, MSC, USN, NAMRU-2

Objective: To find, characterize and evaluate locations in malaria endemic areas of Irian Jaya, Indonesia that can be used for malaria vaccine trials.

Methods: Evaluate the potential of proposed field sites by measuring a set of malaria related parameters including 1) the parasite rate and spleen rate 2) the man-biting rate, sporozoite rate and the entomologic inoculation rate calculated therefrom, 3) the rate of acquisition of new infections by study populations subsequent to radical cure for malaria and 4) the study population size required to show a given level of vaccine efficacy at measured level of transmission pressure.

Progress: We have characterized the transmission of *Plasmodium falciparum* in Arso PIR, Irian Jaya, Indonesia during the high and low transmission seasons. Two groups of adult males were studied, natives of Irian Jaya with lifelong exposure to malaria and Javanese transmigrants with a 2.5 year history of exposure. During the high transmission season (Jan-Apr), 63 transmigrants and 43 Irianese completed the study. Fifty percent of the transmigrants were positive for *P. falciparum* by week 7 after radical cure, 50% of the Irianese by week 9 after radical cure. During the low transmission season study (Jun-Oct), 50% of the transmigrants were *P. falciparum* positive by week 11, and 52% of the Irianese by week 17. *Anopheles koliensis* is the most important vector; the entomologic inoculation rate in January was 0.635 potentially infective bites per man per night, and in April it was 0.029. These data indicate that if transmission remains similar, a vaccine with a protective efficacy of 50% could be shown to be better than placebo (α=0.05, one-tailed, β=0.15) with 60 volunteers in control and vaccine groups during 9 weeks of the peak transmission season, and 17 weeks of the low transmission season.

Plans: Within 30 km of Arso PIR are several other satellite villages which make up the larger Arso complex. Some of these villages contain more recently arrived transmigrants who were evaluated prior to leaving Java for Irian Jaya. Further studies of the type described above will prepare the Arso complex (population ~10,000) for the execution of multiple vaccine trials.

Abstracts:
1. Jones TR, Baird JK, Bangs MJ Annis BA, Purnomo, Basri H, Gunawan S and Hoffman SL. Attack rate and entomologic inoculation rate of *P. falciparum* in Arso, Irian Jaya, Indonesia. XIIIth International Congress for Tropical Medicine and Malaria, Jomtien, Pattaya, Thailand, Nov 92, submitted.

Publications:

1. Jones TR, Baird JK, Basri H, Purnomo and Danidirgo EW. Prevalence of malaria in native and transmigrant populations - Effects of age and history of exposure. Trop Geog Med, 43:1-7, 1991.

2. Baird JK, Jones TR, Danudirgo EW, Annis BA, Bangs MJ, Basri H, Purnomo and Masbar S. Age-dependent acquired protection against *Plasmodium falciparum* in people having two years exposure to hyperendemic malaria. Amer J Trop Med Hyg, 45: 65-76, 1991.

Project Title: Protecting humans against P. falciparum by immunization with a recombinant CS protein and a novel adjuvant, DETOX™.
Funding: 6.3
Principal Investigator: Stephen L. Hoffman, CDR, MC, USNR
Co-Investigators: Trevor R. Jones, LCDR, MSC, USN
Dan Gordon, LTC, USA
Joe Bryan, LCDR, MC, USN

Objective: To determine if immunization with a recombinant CS protein vaccine based on the repeat region in combination with the adjuvant DETOXTM would protect against malaria.

Methods: The Plasmodium falciparum circumsporozoite (CS) protein vaccine, R32NS181, which includes 30 copies of NANP and 2 copies of NVDP, the major and minor tandem repeats of the P. falciparum CS protein, fused to 81 amino acids from the non-structural protein of influenza A, administered with monophosphoryl lipid A, cell wall skeleton of mycobacteria, and squalane (DetoxTM) as adjuvant, was evaluated for safety, immunogenicity, and efficacy in 12 volunteers.

Progress: One volunteer developed a reaction at the injection site after the second dose and declined further immunization. The 11 other volunteers tolerated the 3 doses of 1230 μg vaccine, but most complained of sore arms similar to after typhoid immunization. Two weeks after the third dose of vaccine, sera from four of the eleven volunteers met at least two of the three following criteria: indirect fluorescent antibody titer ≥ 1/4096, anti-R32 antibody level ≥ 15 μg/ml and inhibition of sporozoite invasion of hepatoma cells ≥ 90%. Two of these four individuals were protected against malaria when challenged by the bite of five P. falciparum-infected Anopheles stephensi, and the other two had a delay in the onset of parasitemia (2 days and 5 days) as compared to the volunteers who did not have the same levels of antibodies, and non-immunized controls challenged on the same day. The antibody tests did not discriminate between the two protected individuals and the two who had a delay in onse t of parasitemia. These data indicate that subunit vaccine-induced antibodies against the CS protein can reduce and prevent effective sporozoite invasion of hepatocytes, but that vaccine formulations must be improved so as to consistently induce immune responses capable of protecting against malaria.

Plans: Because of toxicity this adjuvant will not be used in additional studies, the data will be used to formulate subsequent vaccine trials.

Abstracts: Stephen L. Hoffman, Robert Edelman, Joe Bryan, Imogene Schneider, Jonathan Davis, Martha Sedegah, Rafael Harpaz, Dan Gordon, Mitch Gross, Michael Hollingdale, David Clyde, Marcelo Sztein, Scott Paparello, Trevor

Jones. SAFETY, IMMUNOGENICITY, AND EFFICACY OF A MALARIA SPOROZOITE VACCINE ADMINISTERED WITH MONOPHOSPHORYL LIPID A, CELL WALL SKELETON MYCOBACTERIA AND SQUALANE AS ADJUVANT. Annual Meeting of the Am. Soc. Trop. Med. and Hyg., Boston, Mass, Dec 1991.

Publications: Rickman LS et al. 1991. Use of adjuvant containing cell-wall skeleton monophosphoryl lipid A, and squalane in malaria circumsporozoite protein vaccine. Lancet 337:998-1001.

Attacking the sporozoite

Project Title: Safety, immunogenicity, and protective efficacy of synthetic peptide P. falciparum CS protein repeat region vaccines presented as branched chain polymers with a defined tetanus toxoid T helper cell epitope or as long synthetic peptides conjugated to PPD.

Funding: 6.3

Principal Investigator: Preston Church, LCDR, MC, USNR
Co-Investigators: Scott Paparello, LCDR, MC, USNR
Stephen L. Hoffman, CDR, MC,USNR
Dan Gordon, LTC, MC USA
Eniricerche, Milan, Italy
G.P. Corradin, Ph.D., Laussane, Switz.
G. del Guidice, Ph.D., Geneva, Switz.

Objective: To evaluate the safety, immunogenicity, and protective efficacy of two synthetic peptide CS protein vaccines in humans.

Methods: Synthetic peptide vaccines will be constructed using proprietary information. There will be two vaccines. The first will include a P. falciparum CS protein repeat, repeated 40 times and then conjugated to PPD. The second will be a branched chain polymer synthesized including a T helper epitope from tetanus toxoid and a CS protein repeat region peptide.

Progress: Both vaccines have been evaluated in mice and found to be highly immunogenic.

Plans: After completion of a CRDA with Eniricerche the vaccines will be formulated in the U.S in preparation for IND submission. After formulation and pre-clinical testing, the vaccines will be evaluated in clinical trials.

Publications:

Attacking the sporozoite

Project Title: Characterization of the polyclonal antibody response to a four amino acid protective epitope from the circumsporozoite protein of *Plasmodium vivax*.
Funding: 6.1/6.2
Principal Investigator: Trevor R. Jones, LCDR, MSC, USN
Co-Investigators: Yupin Charoenvit, Ph.D.

Objective: To develop methods for inducing a polyclonal antibody response to the four amino acid peptide AGDR from the CS protein of *Plasmodium vivax* such that the polyclonal response mimics the protection provided by an anti-AGDR monoclonal antibody.

Methods: The humoral responses of humans, monkeys and mice immunized with vaccines based on the sequence AGDR were evaluated by ELISA and immunofluorescence for specific activity against AGDR. Sera from human subjects living in a *P. vivax* endemic area were analyzed for naturally induced antibodies to AGDR.

Progress: A monoclonal antibody (NVS3) that passively protects monkeys against sporozoite challenge recognizes a four amino acid linear sequence AGDR included within the repeat region of the *P. vivax* CS protein but when monkeys and human volunteers were immunized with a vaccine, $NS1_{81}V20$, which contains 20 copies of the nonamer, they failed to produce antibodies to AGDR. To determine if natural exposure to sporozoites induces antibodies to AGDR, we tested sera from 176 individuals from a malaria endemic area in Flores, Indonesia. Seventy-one percent of the adults had antibodies to the *P. vivax* repeat region; only 18% had detectable antibodies to AGDR. None of the subjects had antibodies to the *P. vivax* variant repeat, ANGAGNQPG. We subsequently immunized Aotus monkeys and BALB/c mice with AGDR. Sera from the mice reacted strongly with both AGDR and a recombinant protein containing the 20 copies of the nonamer. Sera from the monkeys reacted only minimally with a protein (VIVAX-1) which contains monomeric AGDR within its sequence. Sera from the mice also bound air dried *P. vivax* sporozoites; sera from the monkeys did not. These data indicate that natural exposure induces very low levels of antibodies to the CS protein epitope, AGDR, and that polyclonal antibodies that recognize native CS protein can be induced experimentally with a synthetic AGDR peptide.

Plans: Different AGDR-based sequences (PAGDR, PAGDRA and AGDRA, for example) are now being evaluated for their ability to block the binding of NVS3. In addition a number of synthetic peptide constructs, linear petides including 3-6 repeats of AGDR conjugated to carrier proteins as well as branched polymers including AGDR and a T helper cell epitope from the P. vivax CS protein and from tetanus toxoid are being synthesized and evaluated. The most immunogenic constructions will be used in trials in Saimiri monkeys and if pre-clinical and/or Saimiri monkey studies are promising, will be studied in human volunteers.

Abstracts:

1. Jones TR, Collins WE, Charoenvit Y, Beaudoin RL and Hoffman SL. A four amino acid protective epitope on the circumsporozoite (CS) protein of *Plasmodium vivax*. 39th Annual Meeting Amer Soc Trop Med Hyg, Dec 1-5, 1990.

2. Jones TR, Yuan LF, Marwoto H, Gordon DM, Wirtz RA and Hoffman SL. Natural exposure and immunization with a subunit vaccine do not induce significant antibody levels to a *Plasmodium vivax* protective epitope. 40th Annual Meeting Amer Soc Trop Med Hyg, Dec 1-5, 1991.

Publications:

1. Charoenvit Y, Collins WE, Jones TR, Millet P, Yuan L, Campbell GH, Beaudoin RL, Broderson JR and Hoffman SL. Inability of malaria vaccine to induce antibodies to a protective epitope contained within its sequence. Science, 251:668-671, 1991.

2. Jones TR, Yuan LF, Marwoto HA, Gordon DM, Wirtz RA and Hoffman SL. A *Plasmodium vivax* circumsporozoite protein epitope bound by a protective monoclonal antibody has low immunogenicity. Amer J Trop Med Hyg, in press.

Project Title: Immunogenicity and protective capacity of synthetic peptide vaccines based on the CS protein.

Funding: 6.1/6.2

Principal Investigator: Wang Ruobing Ph.D. (will be on board in July 92)

Co-Investigators: Yupin Charoenvit Ph.D.
Leo Yuan Ph.D.
Martha Sedegah Ph.D.
Stephen L. Hoffman, CDR, MC, USNR
Arnold Satterwait, Ph.D., Scripps Research Institute

Objective: Identify the critical epitope(s) recognized by a protective Mabs against the P. yoelii CS protein and a synthetic peptide based on a QGPGAP repeat sequence; construct, and deliver these subunit vaccines to animal hosts; and, evaluate the protective capabilities of the vaccines including these epitopes.

Previous work has established that passive transfer of monoclonal antibodies against Plasmodium sp. circumsporozoite (CS) proteins protects against challenge with live sporozoites. However, subunit vaccines designed to produce protective polyclonal antibodies against the CS protein have never provided protection comparable to that achieved after passive transfer of Mabs. In these studies we will identify the critical epitopes recognized by the protective Mabs against the P. yoelii CS protein and synthetic peptide based antigen, and construct, deliver, and evaluate the protective capabilities of vaccines including these epitopes. Our main goal is to induce polyclonal antibodies with functional activity similar to protective Mabs. by using vaccines containing appropriate B and T cell epitopes in an appropriate configuration and appropriate antigen delivery system.

Method: P. yoelii subunit vaccine containing B and T cell epitopes in different configurations will be constructed. These antigens will be coupled to appropriate carrier molecules and used in the immunization of mice and rabbits in the presence of appropriated adjuvant, via different routes of administration. Serum antibody levels will be measured in the IFAT against P. yoelii sporozoites, and in the ELISA against the eliciting antigens. Immunized mice will be challenged with P. yoelii sporozoites at the end of the immunization, to determine the protective effect of each formulated subunit vaccines.

Plans: Knowledge gained from these studies will be incorporated in the formulation of human malaria vaccines.

Abstracts: None
Publication: None

Attacking sporozoites (primary)
Attacking infectedhepatoctyes.
Attacking free merozoites.

Project title: Generation of recombinant human monoclonal antibodies for passive protection against malaria

Funding: 6.2

Principal Investigator: William O. Rogers, LCDR, MC, USNR
Co-Investigators: Angray Kang, Ph.D. (Scripps Clinic)
Dennis Hooper, M.D. (Pathology, NAVHOSP, SANDIEGO)
Mitch Gross, Ph.D. (SB Pharmaceuticals).
Yupin Charoenvit, Ph.D.

Objective: To generate human monoclonal antibodies against malaria proteins for use as short-term anti-malarial prophylaxis.

Methods: 1) In collaboration with Mitch Gross (SB Pharmaceuticals), a mouse Mab, NFS2 (Navy falciparum sporozoite 2) that blocks invasion and development of sporozoites in human heptocytes greater than 98% has been humanized by producing a chimeric mouse (variable region) and human (constant regions) IgG, and by inserting the mouse CDRs onto an entirely human framework. 2) In collaboration with Angray Kang (Scripps Clinic) generate combinatorial libraries of antibody genes from the immune response of individuals protected against malaria by immunization with irradiated sporozoites, and from individuals with lifelong exposure to malaria. In this technique, the genes encoding the variable light and variable heavy chain segments of the immunoglobulin gene are amplified by reverse transcription polymerase chain reaction from the circulating B cells of an immunized individual, combined and expressed as a recombinant Fab on the surface of a filamentous phage. Phage containing recombinant Fab with the desired specificity are purified by iteritive cycles of affinity purification on the antigen of interest. Recombinant Fab with desired properties of binding to *P. falciparum* sporozoites and inhibition of sporozoite invasion of hepatocytes in vitro, will be fused to the Fc domain to produce full length human monoclonal antibodies, produced recombinantly under GMP conditions and tested for their ability to confer passive immunity against malaria in humans. 3) In collaboration with Dennis Hooper (Pathology, NAVHOSP SANDIEGO), generate human monoclonal antibodies by standard fusion techniques using circulating B cells from irradiated sporozoite immunized volunteers. Evaluation of human monoclonals as in 1) above.

Progress: Chimeric and humanized NFS2 have been produced and shown to react with recombinant CS protein and sporozoites, and inhibit sporozoite invasion of hepatocytes. Combinatorial antibody libraries have been constructed from the peripheral B cells of an irradiated sporozoite immunized volunteer.

Plans: After further characterization at least e of the Mabs will be chosen for safety, immunogenicity, and efficacy studies in Aotus monkeys and in humans.

In the second two projects, isolation and evaluation of human monoclonal antibodies as described above will be continued.

Abstracts: None

Publications: None

Attacking the sporozoite

Project Title: Interaction between sporozoite and hepatocyte
Funding: 6.1
Principal investigator: Patricia de la Vega, BSc
Co- Investigators: Sylvie Mellouk, PhD
Stephen Hoffman, CDR MC USNR
Yossuf Raviv, PhD, NIH

Objective: Investigate the interaction between the sporozoite and hepatocyte during the process of penetration.

Methods: By specific labelling (125I and activation with photofluor) determinate the membrane proteins involved in this process by SDS PAGE. Purification of remarkable proteins. Production of antibodies. Evaluation of their biological activities in vitro (ILSDA) and in vivo.
Preliminary experiments have to be done in order to check the hepatocytes for viability and susceptibility to sporozoites.

Progress: New start

Plan: By this new labelling technique, determine the parasite antigen expressed on the surface of infected hepatocytes using probes available (Mabs against CSP and SSP2, blood stages antigens)

Abstracts: N/A

Publications: N/A

Attacking sporozoites

Project Title: Inhibition of malaria sporozoite infectivity by sulfated glycoconjugates and potential relevance to vaccine development
Funding: 6.1
Principal Investigator: Samuel J. Pancake, PhD
Co-Investigators: Gordon D. Holt, PhD, NIDDKD, NIH
Sylvie Mellouk, PhD
Stephen L. Hoffman CDR, MC, USNR

Objective: (1) To determine the relative abilities of selected sulfated carbohydrate polymer compounds to bind to malaria sporozoite cicumsporozoite proteins, the primary surface proteins of sporozoites, as hypothesized on the basis of amino acid sequence. (2) To determine the effects of the same compounds on sporozoite infectivity and to investigate the mechanisms by which any such effects may be produced.

Methods: (1) Quantification of the ability of cloned radiolabeled circumsporozoite (CS) proteins to bind various sulfated glycoconjugate (SGC) compounds with high affinity and to cells producing such compounds. (2) Determine the ability of such compounds to inhibit the invasion of mouse hepatocytes *in vitro* by sporozoites and to potentially act as hepatocyte surface receptor sites during invasion. (3) Determine the ability of such compounds to inhibit sporozoite infectivity *in vivo* in mice.

Progress: We have shown that cloned CS protein from *Plasmodium yoelii* has an enhanced affinity for selected SGC compounds attached to Sepharose beads, similar to that shown by thrombospondin, one of a group of several proteins sharing a region of sequence homology and characterized as having high affinities for these compounds. CS proteins contain a similar region of sequence homology. A CHO cell line mutant which produces reduced amounts of sulfated glycoconjugates was observed to bind little CS protein or thrombospondin compared to the wild type. SGC compounds observed to bind CS-protein with the greatest affinity (heparin, fucoidan and dextran sulfate) were also observed to inhibit *P. berghei* sporozoite invasion of hepatocytes *in vitro* and sporozoites were found to bind slectively to the sulfated glycolipid, sulfatide, compared to nonsulfated glycolipids. A subset of these same SGC compounds also inhibited sporozoite infectivity *in vivo*.

Plans: Studies are being undertaken to investigate the olecular mechanisms by which sulfated glycoconjugate compounds inhibit sporozoite infectivity. These will include a further characterization of the general mechanism of action of these compounds, i.e., if they block hepatocyte invasion by binding to sporozoites, as suggested by their ability to bind to CS protein, or by some other mechanism, such as binding to hepatocyte components. The possible involvement of hepatocyte suface SGCs, primarily heparin sulfate, as receptors in sporozoite invasion will be determined. The structural requirements for sulfated glycoconjugate inhibitory activity will be further explored, both *in vitro* and *in vivo*. The ability of

peptides, which correspond to the region of homology of CS protein and are suspected to constitute a SGC binding region, to inhibit sporozoite binding activities will be determined, as will the inhibitory effects of antibodies produced against these peptides.

Abstracts:

1. Pancake SJ, Holt GD, Mellouk S and Hoffman SL. Effect of sulfated polysaccharides on *in vitro* development and infectivity of *Plasmodium berghei* sporozoites. 39th annual meeting, Amer Soc Trop Med Hyg, New Orleans, Nov 90.

Publications:

1. Pancake SJ, Holt GD, Mellouk S and Hoffman SL. Malaria sporozoites and circumsporozoite proteins bind specifically to sulfated glycoconjugates. J Cell Biol, in press.

Attacking sporozoites

Project Title: Identification of cell surface components serving as ligands in the binding and invasion of hepatocytes by sporozoites through the use of a recently developed selective labeling procedure

Funding: 6.1
Principal Investigator: Samuel J. Pancake, PhD
Co-Investigators: Sylvie Mellouk, PhD
Yossef Raviv, PhD, NIDDKD, NIH

Objective: A new technique using a membrane-permeant, photoactivable probe will be used to identify those surface components of *Plasmodium* sp. sporozoites and their hepatocyte targets which undergo interaction during the invasion process. This will involve the incorporation of such a probe, in radiolabeled form, into one cell type, which is then activated to undergo covalent reaction with adjacent membrane proteins. Activation requires close physical association with an activated photofluor which has been incorporated into the surface components of the second cell type. Such an association would be expected to occur during the invasion process or from the selective binding of soluble photofluor labeled compounds to the membrane components of probe labeled cells.

Methods: Membrane proteins of live hepatocytes are to be covalently labeled with ^{125}iodine by incubation with a membrane-permeant, radiolabeled, photoactivable probe (^{125}I-napthyl-azide) followed by incubation with and light activation of photofluor labeled sporozoites using incubation conditions similar to those used in the standard inhibition of liver stage development assay. Analysis of hepatocyte membrane proteins labeled with ^{125}I as a result will be determined using PAGE-autoradiography. Sporozoite surface components and sulfated glycoconjugates will be labeled with fluorescein isothiocyanate and related reagents via reaction with protein amino groups or carbohydrate associated aldehyde groups produced by periodate oxidation. The ability of sulfated glycoconjugates to block molecular interactions between the two cell types will be determined using conditions previously demonstrated by us to block the binding to and invasion of hepatocytes by sporozoites.

Progress: New start

Plans: Initially, the ability of sporozoites with incorporated photosensitizers to activate the iodination of selected membrane components in ^{125}I labeled hepatocytes during binding and invasion will be determined. Subsequently, the effects of those sulfated glycoconjugates able to reduce invasion on the labeling of such hepatocyte membrane components would be determined. In addition, the feasibility of doing the reverse, i.e., the labeling of sporozoites with the probe and hepatocytes with the photofluor, will be determined. The ability of photofluors incorporated into carbohydrate residues, both those on cell surfaces and those contained in exogenously added inhibitory sulfated glycoconjugates, to activate probe labeled cells will be determined. Photofluor modified carbohydrate residures

will be producedby established methods, such as periodate oxidation. The reaction of cells containing membrane incorporated probe with photofluor labeled sulfated glycoconjugates will be of particular interest since these are water soluble, high molecular weight (extracellular) compounds, which we have shown to inhibit the invasion process. Our work suggests this inhibition is via interaction with sporozoite surface components, possibly with a specific region of the circumsporozoite protein of the sporozoite surface. The experiments described should help clarify the molecular mechanisms by which such compounds inhibit the invasion of malaria sporozoites.

Abstracts: None.

Publications: None.

Attacking sporozoites

Project Title: Correlation between antibody affinity and protective activity of sporozoite and peptide-induced monoclonal antibodies to the *Plasmodium yoelii* CS Protein

Funding: 6.1/6.2

Co-Principal Investigators: Mucide Ak, PhD
James H. Bower, LT, MC, USNR

Co-Investigators: Yupin Charoenvit, PhD
Martha Sedegah, PhD
Andrew Lee, PhD
Mark Carter, CPT, MSC, USA
Stephen L. Hoffman, CDR, MC, USNR

Objective: To study the correlation between different antibody properties (serum level, subclass, avidity and affinity) and the protective ability of three different monoclonal antibodies directed against the *P. yoelii* CS protein.

Methods: Three monoclonal antibodies of different subclasses were produced in our laboratory- one by immunizing with irradiated *P. yoelii* sporozoites, and two by immunizing with $(QGPGAP)_4$, the major repeat of the CS protein. We then tested their protective abilities by performing an in-vivo passive transfer experiment, and an in-vitro ILSDA. Serum levels were calculated by ELISA and IFA. Antibody avidity was determined by thiocyanate elution assay, and antibody affinity was determined by a competitive inhibition ELISA.

Progress: All three monoclonal antibodies were found to protect against sporozoite challenge. However, there was a ranking of the three with NYS1 (an IgG3 produced against irradiated sporozoites) being the most protective, QGP-S2 (IgG2b) being the least protective, and QGP-S1 (IgG1) giving a protection level between the two. These results did not correlate with serum levels- all gave the same IFA level, and by ELISA QGP-S2> NYS1> QGP-S1. The results did not correlate with avidity results either (QGP-S2> NYS1> QGP-S1). They did however correlate with the measured affinity levels to (QGPGAP)2, with NYS1 having the highest Ka value followed by QGP-S1 followed by QGP-S2.

Plans: We have found an assay that measures an antibody property that correlates with that antibody's protective efficacy. We plan to use this assay with other antibodies directed against other species and/or stages to determine if the correlation persists.

Abstracts:

1. Ak M, Charoenvit Y, Sedegah M, Lee A, Carter M, Beaudoin RL and Hoffman SL. Lack of correlation between antibody affinity and protective activity of sporozoite and peptide-induced monoclonal antibodies to the *Plasmodium yoelii* CS protein. IV International Congress on Malaria and Babesiosis, Rio de Janiero, Brazil, 13-17 Aug, 1990.

Attacking sporozoites

Project Title: Characterization of a monoclonal antibody to a second epitope within the repeat sequence of the *Plasmodium vivax* circumsporozoite protein.

Funding: 6.1/6.2

Principal Investigator: Yupin Charoenvit, PhD
Co-Investigators: James Bower, LT, MC, USNR
Leo Yuan, PhD
Trevor R. Jones, LCDR, MSC, USN
Stephen L. Hoffman, CDR, MC, USN
Victoria Fallarme

Objective: Our goal is to develop a malaria vaccine that induces immune responses against multiple targets on the CS and other malaria proteins. To accomplish this goal we produced a series of monoclonal antibodies (Mabs) to *P. vivax* sporozoites and used them in identifying target antigens that may be used as vaccine candidates.

Methods: Mabs directed against *Plasmodium vivax* sporozoites have been produced. NVS3 (Navy vivax sporozoite 3) and NVS4 (Navy vivax sporozoite 4) were selected for further studies. In previous studies, NVS3, an IgG3 Mab protected monkeys against *P. vivax* sporozoite challenge in passive transfer experiments, and this Mab recognized four (AGDR) of the nine (GDRADGQPA) amino acids from the repeat region of the *P. vivax* CS protein (1). In this study, NVS4, an IgG1 Mab will be immunologically and functionally characterized.

Progress: NVS4 recognizes air-dried sporozoites in an IFAT, precipitates the surface coat of live sporozoites, and recognizes yeast and *E. coli*-produced *P. vivax* CS protein and synthetic $(GDRADGQPA)_2$ in ELISA. Like NVS3, it does not react with *P. falciparum, P. berghei, P. yoelii,* or *P. gallinaceum* sporozoites. However, our preliminary data shows that NVS4 recognizes an amino acid sequence of the repeat region of the *P. vivax* CS protein distinct from that recognized by NVS3. NVS4 does not recognize $(AGDR)_2$ in ELISA, and its recognition of CS protein by ELISA is not inhibited by $(AGDR)_2$, AGDRA, PAGDR, or PAGDRA. Epitope mapping and characterization of affinity and functional activity of NVS4 is in progress.

Plan: Purified NVS4 will be tested for a protective effect in the inhibition of liver stage development assay (ILSDA), and by passive immunization against *P. vivax* sporozoites in monkeys. Epitope mapping of NVS4 is underway. If NVS4 is protective, the epitope recognized by NVS4 may be very important in malaria vaccine formulation.

Abstract:

1. Charoenvit Y, Bower J, Yuan L, Jones TR, and Hoffman SL Characterization of a monoclonal antibody to a second epitope within the repeat sequence of the *Plasmodium vivax* circumsporozoite protein. XIIIth International Congress for Tropical Medicine and Malaria, Jomtien, Pattaya, Thailand, Nov 92.

Publications:

1. Charoenvit Y, Collins WE., Jones TR, Millet P, Yuan L, Campbell G.H, Beaudoin RL, Broderson JR and Hoffman SL. Inability of malaria vaccine to induce antibodies to a protective epitope within its sequence. Science 251: 668-671, 1991.

2. Charoenvit Y, Mellouk S, Cole C, Bechara R, Leef MF, Sedegah M, Yuan LF, Robey FA, Beaudoin RL and Hoffman SL. Monoclonal, but not polyclonal, antibodies protect against *Plasmodium yoelii* sporozoites. J Immunol 146: 1020-1025, 1991.

Project Title: Cooperative binding of a NVS3 a murine IgG3 monoclonal antibody to the circumsporozoite protein of *Plasmodium vivax.*

Funding: 6.1

Principal Investigator: Yupin Charoenvit, PhD

Co-Investigators: Laurence J. N.Cooper, PhD, Case Western Reserve University School of Medicine
Leo Yuan PhD
Trevor R. Jones, LCDR, MSC, USN
Neil Greenspan, PhD, Case Western Reserve University School of Medicine
Pascal Millet, PhD, Malaria Branch, Centers for Disease Control
Stephen L. Hoffman, CDR, MC, USNR

Objective: Our previous report showed that NVS3, an IgG3 Mab directed against *P. vivax* CS protein protected monkeys against sporozoite challenge. Other reports showed that IgG3 Mab enhance the strength of binding of IgG to Streptococcal group A carbohydrate. In this study we will determine whether or not the H and C domain of NVS3 influences the strength of binding of radioactive labelled of NVS3 (IgG3) or NVS4 (IgG1) to *P. vivax* CS protein. Information gaining from this study will be extremely valuable in the vaccine formulation.

Method: Varying concentrations of ^{125}I-labelled NVS3 were tested against *P. vivax* antigens coated on wells of polyvinyl chloride microtiter plates, in the presence or absence of unlabelled NVS3 (IgG3), NVS4 (IgG1) or a control Mab. The amounts of radio-labelled NVS3 bound to the plates were counted and the unlabelled antibody was assessed for the inhibitory or enhancement effect on the binding of labelled NVS3 to *P. vivax* antigens. Antigens used in this studies were: VIVAX-1, a recombinant *P. vivax* CS protein containing 20 repeats of nine amino acids GDRADGQPA; $(AGDR)_6$, a synthetic peptide containing 6 repeats of AGDR, an epitope for NVS3, a protective Mab; and *P.vivax* sporozoite antigen extract).

Progress: We are now show that unlabeled NVS3, and IgG3 kappa Mab specific for the CS protein of the *P. vivax*, enhanced the binding of radilabeled NVS3 to solid phase recombinant CS protein, VIVAX-1. Unlabeled NVS4, an IgG1 Mab specific for *P. vivax* CS protein, or unlabeled irrelevant IgG3 mab dose not enhance the binding of labeled NVS3 to VIVAX-1 antigen. Binding of radiolabeled $F(ab')_2$ fragments of NVS3 to VIVAX-1 was not enhanced by unlabeled NVS3 or its $F(ab')_2$ fragments. The gamma 3 FC region is probably involved in the cooperative binding of IgG3 antibody to its specific antigen but additional mechanisms may also be contributing to cooperativity. We are now in the process of switching isotype of NVS3 Mab. The NVS3 containing a new isotype will be studied further for its protective effect, immunological and physical (intrinsic affinity) properties.

Abstracts: None

Publications:
1.Cooperative binding of a NVS3 a murine IgG3 monoclonal antibody to the circumsporozoite protein of *Plasmodium vivax.* manuscript in preparation.

Attacking sporozoites

Project Title: Antibody responses of humans to candidate *vivax* vaccine antigens
Funding: 6.2
Principal Investigator: Eileen D. Franke, LCDR, MSC, USN
Co-Investigator: Ervin San Roman, MD (Occidental Petroleum Corporation of Peru)

Objective: To study the antibody responses of residents of a malarious area in Peru to recombinant and synthetic proteins representing the circumsporozoite (CS) protein and blood stage antigens of *P. vivax*.

Methods: A preliminary malaria prevalence stody was done in approximately 300 children and adult residents of four different villages (Andoas Nuevo, Andoas Viego, Los Jardines and Titiyacu) near the Occidental Petroleum Corporation basecamp in Andoas, Peru. Blood films were prepared and serum samples were obtained. The prevalence of malaria and IgG antibodies to five recombinant and synthetic vivax CS proteins (Vivax-1, -2 and -3, $NS1_{81}V20$ and Pvk247) were determined. *Plasmodium vivax* was the only malaria species infecting humans in this area. The second phase of the project was a three-year malaria incidence study in which approximately 150 children between the ages of 2 and 18 were enrolled. Thick and thin blood film slides were prepared every two weeks following radical cure with chloroquine and primaquine. The IgG and IgM antibody responses to Vivax-2 and Pvk247 were measured using an ELISA. Antibody titers to *P. vivax* blood-stage antigens were determined using an immunofluorescence assay (IFA). The data obtained from this study will provide a better understanding of the magnitude and duration of antibody responses to specific vivax sporozoite and blood-stage antigens in individuals naturally exposed to malaria. This information will be useful in the selection of candidate sporozoite and blood-stage antigens for vaccines. Epidemiological and immunological data collected from the incidence study population will be useful in the event that the area is chosen as a future site for field testing a *vivax* malaria vaccine.

Progress: In the initial study of the prevalence of malaria and antibody responses we found that a higher percentage of the sera had antibodies that recognized Vivax-2 and Vivax-3, the two proteins containing the longest non-repeat sequences, than to $NS1_{81}V20$ or Vivax-1. Children less than 5

Plans: Serum samples obtained from the volunteers over the three year period of the study have been stored at -70°C and will be tested for antibodies to peptides representing non-CS protein sporozoite and blood stage antigens when they become available.

Abstracts:

1. Franke ED, Lucas C, Covenas H and San Roman E. Humoral immune response to the circumsporozoite protein of *Plasmodium vivax* in residents of several village in the Peruvian jungle. 38th Annual Meeting, Amer Soc Trop Med Hyg, 1989.
2. Franke ED, Lucas CM, Cachay M, Covenas H and Wirtz RA. Prevalence of

antibody to the variant repeat of the CS protein of *Plasmodium vivax* in Peru. 40th Annual Meeting, Amer Soc Trop Med Hyg, 1991.

Publications:

1. Franke ED, Lucas CM, San Roman E and Wirtz RA. Prevalence of antibody to the variant repeat of the circumsporozoite antibody of *Plasmodium vivax* in Peru. Amer J Trop Med Hyg, 46: 708-710, 1992.
2. Franke, ED, Lucas, CM and San Roman E. Antibody response of humans to the circumsporozoite protein of *Plasmodium vivax*. Infect Immun 59:2836-2838, 1991.

Project Title: Characterization of the human and murine immune response to 3D7 *P. falciparum* Exported Protein - 1 (Exp-1)
Funding: 6.1/6.2
Principal investigator: Gloria I. Sanchez, M.Sc.
Co-Investigator: Sylvie Mellouk, Ph.D.
William O. Rogers, LCDR MC USNR

Objective: To evaluate the cellular and humoral immune response of humans and mice immunized with irradiated sporozoites against the recombinant Exp-1 protein. Determine the expression of this antigen in human hepatocytes infected with *P. falciparum.*

Methods: 1) To clone and express the exp-1 gene of the 3D7 clone of *P. falciparum* . 2) Characterize the expression of Exp-1 in *P. falciparum* infected hepatocytes. 3) Evaluate the immune response of volunteers immunized with irradiated sporozoites against recombinant Exp-1. 4) Clone and express Exp-1 in P815 cells and evaluate the cellular and humoral immune response of mice immunized with irradiated *P. falciparum* sporozoites.

Progress: (Exp-1) is a blood stage antigen associated with the parasitophorous vacuole membrane which in some isolates contains a region of 15 amino acids homologous to the tandemly repeated NANP epitope of CS protein.(Hope et al. [1985] *Nuc. Aci. Res.* 13, 369-379). The monoclonal antibody, 5.1, that recognizes this antigen in parasitized red blood cells reacts with sporozoites (Hope et al. [1984] *Nature.* 308, 191-194) and liver stage parasites, and inhibits the transformation of sporozoites into liver stages. We have cloned the Exp-1 gene of the 3D7 clone of *P. falciparum* and find that its sequence does not contain the NANP sequence. As expected, mAb 5.1 does not recognize erythrocytes infected with the 3D7 clone. Recombinant 3D7 Exp-1 expressed in *E. coli* is recognized by mAbN1 (Gunther et al. [1991] *Mol. Bioch. Parasitol* 46, 149-158), a monoclonal antibody which specifically recognizes an epitope in the N terminus region of Exp-1 distinct from the NANP epitope recognized by mAb 5.1. Sera raised against the 3D7 Exp-1 will be useful in evaluating hepatic stage expression of Exp-1 because the 3D7 Exp-1 lacks the NANP epitope responsible for cross reaction with the CSP and therefore any fluoresence of infected hepatocytes or activity in the ILSDA assay detected with the anti-3D7 Exp-1 serum will not be attributable to cross reaction with the CSP. A recombinant fusion protein including the entire Exp-1 has been produced.

Plans: Generate antiserum against expressed, recombinant Exp-1 and evaluate the expression of this antigen in liver stages. Evaluate the capacity of this antiserum to inhibit the trasnsformation of sporozoites into liver stages by the Inhibition of Liver Stage Development Assay (ILSDA). Assess the humoral, proliferative T cell, and cytotoxic T cell responses to Exp-1 among human volunteers immunized with irradiated *P. falciparum* sporozoites. Characterize the mouse cytotoxic T cell response against Exp-1 protein in mice immunized

with *P. falciparum* sporozoites.

Publications: Mellouk S, Berbiguier N, Druihle P, Sedegah M, Galey B, Yuan L, Leef M, Charoenvit Y Paul C, Hoffman S and Beaudoin R. Evaluation of an in vitro assay aimed at measuring protective antibodies against sporozoites WHO Bulletin 68: 52-59, 1990.

Project Title: Targeting the circumsporozoite protein for processing in the Class I Antigen presentation pathway
Funding 6.1
Principal Investigator: Miriam D. Rogers, M.D.
Co-Investigator: William O. Rogers, LCDR, MC, USNR

Objective: To develop methods for targetting malaria vaccine antigens to the Class I antigen presentation antigen in order to induce strong cytotoxic T cell (CTL) response against malaria infected hepatocytes.

Methods: Constuction of modified CSP genes and their expression in vaccinia.

Progress: New project

Plans: Induction of CD8+ CTL requires presentation of antigen in the context of Class I MHC molecules. It is currently believed that the pathway to Class I presentation involves degradation of newly synthesized antigen in the cytosol, transport of peptide degradation products from the cytosol to the endoplasmic reticulum by peptide transporters, and association of these short peptides with MHC Class I molecules in the ER. One potential advantage of live viral vaccine vectors is that the foreign gene is synthesized within a host cell and may therefore be more readily targeted for Class I presentation. However, since most candidate vaccine antigens are membrane proteins, which have hydrophobic leader sequences targetting them for cotranslational transport to the ER, these proteins may not be optimally accesible to cytosolic proteases and may not therefore be efficiently presented for induction of CTL. Proteins which bear a destabilizing amino acid at the N-terminus and an internal lysine residue close to the N-terminus are targeted for ubiquitination and subsequent proteolytic degradation in the cytosol. We are therefore constructing recombinant versions of the gene encoding the CSP containing combinations of the following modifications: 1) Removal of the hydrophobic leader and membrane anchor sequences, to prevent cotranslational transport of newly synthesized CSP out of the cytoplasm 2) N-terminal fusion of the complete ubiquitin sequence to the truncated CSP. Ubiquitin is efficiently cleaved from the N-terminus of chimeric proteins to expose any desired amino acid, generating a truncated CSP with either a destabilizing or stabilizing amino acid at the N-terminus 3) Insertion of a fragment of the lac repressor protein between ubiquitin and the truncated CSP. This protein fragment has been shown to contain internal lysine residues which are highly efficient substrates for ubiquitination. When introduced into mammalian cells, these constructs are expected to generate modified CSP targeted for rapid degradation in the cytoplasm and targeting to the Class I antigen presentation pathway. Constructs containing combinations of the above modifications will be introduced into vaccinia and tested for 1) ability of recombinant vaccinia infected cells to serve as targets for CTL using effector cells from irradiated sporozoite immunized mice and 2) ability of the recombinant vaccinia to induce protective CTL in mice. If experiments in the *P. yoelii* CS protein model system are

successful, similar constructs will be made for the *P. yoelii* SSP2, *P. yoelii* MSP-1, and *P. yoelii* Exp-1, and for their *P. falciparum* analogues.

Abstracts: None

Publications: None

Attacking the sporozoite (secondary)
Attacking the infected hepatocyte (primary)

Project Title: Development of PfSSP2 as a vaccine for *P. falciparum* malaria.
Funding: 6.2
Principal Investigator: William O. Rogers, LCDR, MC, USNR
Co-Investigator: Enzo Paoletti, Ph.D., Virogenetics
David Lanar, Ph.D., WRAIR
Luther Lindler, Ph.D., WRAIR
Anita Malik, Ph.D.
Ben Wizel, Ph.D.

Objective: To express PfSSP2 in the live vaccine vectors, vaccinia, *Salmonella* sp., and BCG, to test these constructs for their expression of PfSSP2, their ability to induce humoral and cellular immune responses against PfSSP2, and their ability to provide protection against malaria.

Methods: 1) In collaboration with Enzo Paoletti (Virogenetics) and David Lanar (WRAIR) generate recombinant vaccinia virus expressing the gene encoding PfSSP2 from the 3D7 clone of *P. falciparum*. Detect expression of PfSSP2 with previously generated mouse antisera against PfSSP2. Infect EBV-transformed lymphocytes from irradiated sporozoite immunized human volunteers for use as targets and stimulators in CTL assays to demonstrate the presence of a human CTL response against PfSSP2 in irradiated sporozoite immunized humans. Immunize mice with recombinant PfSSP2-vaccinia to determine if such immunization induces a CTL response in mice. Carry out trials of safety, immunogenicity and efficacy of the recombinant vaccinia vaccine in humans. 2) In collaboration with Luther Lindler (WRAIR), express PfSSP2 in attenuated *Salmonella*. Immunize mice with *Salmonella* expressing PfSSP2 to determine if such immunization induces a CTL response in mice. Carry out trials of safety, immunogenicity, and efficacy in humans.

Progress: Constructed appropriate plasmid for expression of PfSSP2 in *S. typhimurium*. Succesful expression of a fusion protein including amino acids 145-574 of PfSSP2 and the 260 amino acid leader of phage T7 gene 10. Constructed plasmid for recombination into vaccinia including the full length PfSSP2 gene.

Plans: Continue evaluation of expression and immunogenicity in the vaccinia and Salmonella systems as described above.

Abstracts: None

Publications: None

Attacking the sporozoite (secondary)
Attacking the infected hepatocyte (primary)

Project title: Characterization of the gene encoding *Plasmodium yoelii* SSP2
Funding: 6.1
Principal Investigator: William O. Rogers, LCDR, MC, USNR
Co-Investigators: Miriam D. Rogers, M.D.
Richard Hedstrom, LCDR, MSC, USN

Objective: To characterize the gene encoding the *Plasmodium yoelii* sporozoite surface protein 2.

Methods: Use a monoclonal antibody against PySSP2 to isolate the gene from a *P. yoelii* genomic expression library. Sequence the gene. Use reverse transcription polymerase chain reaction and Northern blotting to identify transcription of the gene in pre-erythrocytic and erythrocytic stages.

Progress: The complete sequence of the gene encoding *P. yoelii* SSP2 has been determined. The coding sequence consists of a single, long open reading frame encoding 826 amino acids. The overall structure of SSP2 is similar to that of the CSP, consisting of a central region of immunogenic amino acid repeats flanked by non-repetitive sequence. SSP2 has one copy of a thrombosponding repeat motif in common with several cell adhesion molecules as well as with the CSP and the Thrombospondin Related Anonymous Protein of *P. falciparum*. Additionally, SSP2 shares substantial sequence similarity to TRAP, suggesting that TRAP is the analog of SSP2 in P. falciparum. Expression of SSP2 in pre-erythrocytic stages was demonstrated by reverse transcription PCR of RNA from infected mosquitoes.

Plans: Characterize blood stage expression of SSP2 by reverse transcriptase PCR and Northern analysis.

Publications:
1. Charoenvit Y, Leef MF, Yuan LF, Sedegah M and Beaudoin RL. Characterization of *Plasmodium yoelii* monoclonal antibodies directed against stage-specific sporozoite antigens. Infect Immun 55,604-608, 1987

2. Hedstrom RC, Campbell JR, Leef ML, Charoenvit Y, Carter M, Sedegah M, Beaudoin RL and Hoffman SL. A malaria sporozoite surface antigen distinct from the circumsporozoite protein. Bull World Health Org. 68 (Suppl.):152-157, 1990

3. Rogers WO, Rogers MD, Hedstrom RC & Hoffman SL. Characterization of the gene encoding sporozoite surface protein 2, a protective *Plasmodium yoelii* sporozoite antigen. Mol Biochem Parasitol 53:45-52, 1992

Attacking sporozoites.
Attacking infected hepatocytes (primary)

Project Title: Determination of the immune mechanisms involved in irradiated sporozoite induced cross protection against *P. berghei* and *P. yoelii* sporozoites in BALB/c mice.

Funding: 6.1

Principal Investigator: Martha Sedegah, PhD
Co-Investigators: Walter R. Weiss, CDR, MC, USN
Sylvie Mellouk, PhD
Stephen L. Hoffman, CDR, MC, USNR

Objectives: To determine if cross protection exists between *P. berghei* and *P. yoelii* and to determine the immune mechanisms involved in the cross immunity.

Methods: BALB/C mice are immunized by multiple injections of irradiated sporozoites of *P.berghei* (ANKA clone) or *P. yoelii* (17NL, 1.1 clone). Protection is measured as absence of blood parasitemia after challenge with homologous or heterologous infective sporozoites. T-cell subset depletions experiments are carried out by injecting immunized mice with monoclonal antibodies to either CD4+ or CD8+ T-cells and confirming the depletions by FACS analysis. Immunized and T-cell subset depleted mice are rechallenged with homologous or heterologous infective sporozoites. Levels of antibodies to sporozoites of both parasites are determined by IFAT.

Progress: BALB/c mice immunized by multiple injections of irradiated sporozoites of *P.berghei* (ANKA) or *P. yoelii* (17NL, 1.1 clone) exhibited a significant level of cross protection between the two malaria species. Protection was stage specific and did not extend to challenge with erythrocytic stages. In depletion experiments, we found that depleting *P. berghei* immunized mice of their CD8+ T cells aborogated their immunity to *P. yoelii* but not to *P. berghei* challenge. Depleting *P. berghei* immunized mice of their CD4+ T-cells diminished their immunity to *P. yoelii* but not to the homologous *P. berghei* challenge. When the *P. berghei* immunized mice were depleted of both their CD4+ and CD8+ T-cells, protection against *P. berghei* i was slightly reduced. However, depleting *P. yoelii* immunized mice of their CD8+ T-cells abborogated their immunity to both *P. berghei* and *P. yoelii*. In all experiments *P. yoelii* sporozoites were more than 100 times as infective in BALB/c mice as *P. berghei* sporozoites.

Plans: We are immunizing mice with irradiated sporozoites of *P.yoelii,* depleting them of their CD4+ T-cells and then challenge them with the heterologous parasite *P. berghei* .

Publications: Manuscript in preparation.

Attacking the sporozoite
Attacking the infected hepatocyte (primary)

Project Title: Immunological characterization of P. yoelii Sporozoite Surface Protein 2
Funding: 6.2
Principle Investigator: Helen H. Wang, M.D.
Co-Investigators: Yupin Charoenvit , PhD
William O. Rogers, M.D., PhD

Objective: To localize regions of P.yoelii SSP2 responsible for the induction of protective cell mediated immune response against malaria.

Methods: SSP2 is a 140 kDa sporozoite surface protein. Its amino acid sequence is characterized by a central region of short amino acid repeat units flanked by non-repetitive sequences on both the amino and carboxy terminii. Experiments carried out by Dr. Khusmith demonstrated that immunization with a fragment of SSP2 including the central repeats and a small amount of the flanking, non-repeated sequence, when delivered in mouse mastocytoma transfectants expressing the SSP2 fragment, could provide partial protection against P. yoelii malaria [Khusmith et al. Science 252:715-718]. This project is designed to determine first, whether immunization with the full length SSP2 protein provides similar or better protection than that provided by Khusmith's original fragment and, second, to identify which regions of SSP2 are responsible for the induction of protective cell-mediated immunity. The following approach is being taken: 1) Cloning complete open reading frame of SSP2 and truncated versions including the repeats alone, the repeats plus the amino terminal non-repeated sequence, and the repeats plus the carboxy terminal non-repeated sequence, into the mammalian cell expression vector, pCEXV3; 2) Transfecting each of them into mousee mastocytoma P815 cells; 3) Testing antigen expression by PCR, IFA, and Western blotting; 4) Determining whether the transfectants will function as targets for killing by effector cells from irradiated sporozoite immunized mice,and 5) Immunizing mice with transfectants and evaluating the protection against malaria.

Progress: . The complete open reading frame of SSP2 and the truncated form including the repeats and the carboxy terminal non-repeated sequence have been cloned into pCEXV3 and transfected into P815 cells. The expression of full length SSP2 and the C-terminal fragment has been demonstrated by IFA, DNA PCR and Western blot. Immunization of mice with full length SSP2 transfectants has begun.

Plan: After five immunizations, mice will be tested for the presence of anti-SSP2 antibodies and for protection against malaria. CTL assays using effector cells from irradiated sporozoite immunized mice and the transfectants as targets will be carried out. The results will be compared with the results originally obtained with Khusmith's partial SSP2 transfectant, and other truncated fragments of SSP2 will be tested as they are produced.

Abstract: None

Publication: None

Attacking the infected hepatocyte (primary)

Project Title: Characterization of cultured mouse hepatocytes expressing the P. yoelii surface proteins, CSP and SSP2

Funding: 6.1

Principle Investigator: Helen H. Wang, MD
Co-Investigators: Samuel I. Pancake, PhD
Sylvie Mellouk, PhD

Objective: To express the P. yoelii pre-erythrocytic stage antigens CSP and SSP2 in hepatocytes, the natural host for the pre-erythrocytic stage of Plasmodium, and to determine whether that expression will make hepatocytes effective targets for anti-malarial cytotoxic T cell activity. To study the cell-cell binding specificities of hepatocytes expressing CSP or SSP2.

Methods: 1) Cloning P. yoelii CS and SSP2 gene into the mammalian cell expression vector pREP9 ; 2) Transfecting the DNA into an SV40 transformed, BALB/C mouse hepatocyte cell line; 3) In vitro expression of the antigens are tested by IFA, DNA and RNA PCR, Western blot; 4) Test for CTL activity using the mouse hepatocyte transfectants as targets and cells from sporozoite immunized mice as effectors 5) In vitro infecting the same hepatocyte with sporozoites; 6) Assays for cell binding to sulfated glycoconjugate coated substrates to determine if transfected cells have altered binding specificities.

Progress: P. yoelii CS and SSP2 protein gene have been cloned into the mammalian cell expression vector, pREP9. Prior studies have shown that both the CSP and SSP2 are targets of a CTL response in sporozoite immunized mice. It has been shown that the P. yoelii CS protein can selectively bind to sulfated glycoconjugate compounds, such as heparin. This binding may be related to a short nonapetide sequence homologous to a thrombospondin binding motif; such a sequence is also found in SSP2, suggesting that transfected cells expressing CS or SSP2 on their surfaces may show modified adherence properties.

Plan: Transfect SV40 transformed BALB/c mouse hepatocyte cell line with P.yoelii CS and SSP2 gene, select the clones and demonstrate expression as above. Assay for CTL response against the transfectants in irradiated sporozoite immunized mice. Determine the ability of transfected cells expressing sporozoite proteins on their surfaces to adhere to plastic substrates coated with sulfated glycoconjugate conpounds, and if binding is demonstrated, to determine the effects of addition of CS and SSP2 proteins and peptides and antibody against on binding.

Abstract: None

Publication: None

Attacking the sporozoite
Attacking the infected hepatocyte (primary)

Project Title: Expression of malaria proteins CSP and SSP2 in Leishmania parasites
Funding: 6.2
Principal Investigator: Helen H. Wang , MD
Co-Investigators: William O. Rogers, MD, PhD
Dyann Wirth, PhD, Harvard School of Public Health

Objective: To use Leishmania parasites expressing thePlasmodium proteins, CSP and SSP2, as a vaccine against malaria.

Methods: 1) Cloning P. yoelii circumsporozoite (CSP) gene and Sporozoite Surface Protein 2 (SSP2) gene into a Leishmania parasite expression vector pALT-neo; 2) transfecting by electroporation the DNAs into Leishmania enreittii and major; 3) testing protein expression by immunofluorescence (IFA) , DNA and RNA PCR, and Western blotting; 4) Immunizing mice with malaria protein transfected Leishmania parasites and evaluating the protection against malaria.

Progress: Leishmania is an intracellular protozoal parasitewhich causes cutaneous ulcers. Species specific cell mediated immunity to leishmaniasis occurs following cure of the lesions. Leishmania infection induces both CD8+ and CD4+ anti-leishmanial CTL activity. It seems possible that infection with a Leishmania strain expressing malaria proteins could induce anti-malarial cell mediated immunity. A Leishmania expression vector, pALT-neo, was supplied by Dr. Wirth's lab. The P. yoelii CSP and SSP2 genes have been cloned into pALT-neo. Experiments to introduce these plasmids into L. enriettii are underway.

Plans: The expression of P. yoelii CS and SSP2 proteins in L. enriettii will be tested by both DNA and RNA PCR , IFA, and Western blotting. If expression is succesful, the plasmids will be introduced into L. major and the transfectant parasites used to immunize mice. These mice will be tested for the presence of anti CSP and SSP2 antibodies andCD4+ and CD8+ anti-malarial CTL, as well as for protection against malaria.

Abstracts: None

Publications: None

Attacking the infected hepatocyte (primary)

Project Title: Plasmodium yoelii hepatic stages cDNA library
Funding: 6.1
Principal investigator: Patricia de la Vega, BSc
Co-Investigators: Sylvie Mellouk, PhD
Stephen Hoffman MD

Objective : Construction of cDNA substraction library of P. yoelii hepatic stages.

Methods : Preparation of mRNA from normal and P. yoelii infected hepatocytes. Construction of an in vitro infected hepatocyt cDNA library. Use the mRNA from normal cells to construct a subtraction library.

Progress: New start

Plan: Preliminary experiments are going to be done using an in vitro rodent model. If the model is available, the goal is to work with a human P. falciparum model.

Abstract: N/A

Publications: N/A

Attacking the infected hepatocyte (primary)

Project Title: Hepatic stages: Cellular Immunity
Funding: 6.1
Principal investigator: Sylvie Mellouk, PhD
Co- Investigators: Patricia de la Vega
Stephen Hoffman, MD
Dr.M. Patt, NIH
Dr. D. Margulies, NIH

Objective: Determination of the epitope expressed on the surface of infected hepatocyte and involved in the cytotoxic killing.

Methods: Immunization of BALB/c mice with P. yoelii irradiated-sporozoites. Boost in vitro the immune spleen cells with in vitro (24h-48h) P. yoelii infected hepatocytes used as extract or fixed with glutaraldehyde. Evaluation of cytotoxic effect of those cells in vitro infected cultures (P. yoelii, P. bergheii) of BALB/c and BALB/k hepatocytes (antigenic specificity and MHC restriction).

Progress: New start

Plan: So far, the research has been focused on the sporozoite level to findthe epitopes expressed on infected hepatocytes (which are the target for the cellular immunity on irradiated-sporozoite vaccine). By looking directly at the hepatic level, it would be maybe possible to determinate the epitopes which are certainly multiple (sporozoite epitope but also specific of hepatic stage). The goal of this study is 1. to demonstrate the capacity of EE stages to boost immunocompetent cells. 2. to eluate by HPLC infected and non hepatocytes. 3. to test the different eluated fractions with immune spleens cells on 51Cr assay on direct infected hepatocytes. 4. to analyze positive fraction and synthesize peptides.

Abstracts: N/A

Publications: N/A

Attacking the infected hepatocyte (primary)

Project Title: Hepatic stages: humoral immunity
Funding: 6.1/6.2
Principal investigator: Sylvie Mellouk, PhD
Co-Investigators: Mucide Ak, PhD, James Bower, MD, Yupin Charoenvit, PhD
Patricia de la Vega, BSc, Stephen Hoffman, MD
William Rogers, MD, PhD,
Gloria Sanchez MSc
Martha Sedegah, PhD
Dr. Masamichi Aikawa, Institute of Pathology Case Western Reserve University
Dr. M. Gross, SmithKline Beecham
Dr. A. Nussler, University of Pittsburg

Objective: To evaluate the in vitro activities of antibodies directed against pre-erythrocytic stages (sporozoites, liver stages) and blood stages. To characterize the presence of those antigens during the parasite development.

Methods: Use of in vitro human and rodent models (primary cultures of human or rodent hepatocytes inoculated with human or rodent malaria sporozoites) which takes into account not only the penetration but also the development of the parasite in its normal host cell. Evaluate the inhibitory activities of those antibodies (Mabs or antisera) directed against defined antigens (penetration or post-penetration). Characterize the presence of those antigens by IFA, EM, and Western blot during hepatic stage development.

Progress: This in vitro model has been used :
1) To evaluate the activities of:
- Mab directed against P. falciparum CSP or P.yoeliii CSP and SSP2 or P. yoeliii EE stage antigen NYLS3.
- Immune mouse sera from mice immunized with different P. yoeliii vaccine (X-Spz, passive transfer sera from mice immunized with Proteosome P. yoeliii CS, Mabs directed against various epitopes of the P. yoeliii CSP or P. falciparum SSP2.
- Immune human sera immunized with P.falciparum CSP (R32tet32-Detox vaccine)
2) To characterize the presence of the:
- P.yoelli CSP, SSP2, NYLS3 during the course of the P. yoelii hepatic stage(6h, 24, 48, 72h) by IFA and EM using Mabs.
- P. falciparum SSP2 during the course of P. falciparum hepatic stage (48h, 4, 5 6 days) by IFA using immune sera.

Plans: From these different data, if there is a correlation between the ILSDA and the in vivo observations, it is clear that this test cannot predict the protective capacity of one specific vaccine. However, ILSDA provides a meaninful test in the evaluation of the antibody component of the immune response by comparison with

others tests (IFA, ELISA). The culture of hepatic stage, particularly P. falciparum (the development of this human parasite only occurs in human hepatocytes), is an useful tool for the characterization, localization and determination of one specific antigen (P. falciparum and P. yoelii blood stage antigen are now under investigation). Some preliminary experiments seem to indicate that antibody directed against hepatic schizont can interfere with the parasite development. More extensive experiments have to be set up to confirm this result. An ADCC mechanism with anti-P. yoeliii liver stage is under investigation. Finally, we plan to study if there is a blood stage feed-back on the hepatic stage (direct or indirect).

Abstract: 1. Charoenvit Y, Mellouk S, Sedegah M, Leef M, de la Vega P, and Hoffman S. Characterization of an inhibitory antibody against P. yoelii liver stage parasites. 40th meeting, Amer Soc Trop Med Hyg, Boston, MA, Dec 1-5, 1991

Publication: 1. Charoenvit Y, Mellouk S, Cole C, Bechara R, Leef MF, Sedegah M, Yuan LF, robey FA, Beaudoin RL and Hoffman SL. Monoclonal, but not polyclonal, antibodies protect against Plasmodium yoelii sporozoites. J Immunol 146:1020-1025, 1991.

Attacking the infected hepatocyte (primary)

Project Title: Hepatic stages: non specific cellular immunity
Funding: 6.1
Principal investigator: Sylvie Mellouk, PhD
Co-Investigators: Dr. A. Nussler, Department of Surgery, University of Pittsburgh
Dr. S. Green, Dr. C. Nacy, Walter Reed Army Institute of Research

Objective: In vitro effect of cytokines (i.e. IFNg, IL6, TNF) on P. berghei , P. yoelii and P. falciparum hepatic stages. Mechanism of killing: role of nitric oxide (NO). Do the parasite act as second activation signal for the NO synthesis of IFNg-treated infected hepatocyte? Importance of cytokines in the irradiated-sporozoite vaccine.

Methods: In vitro inhibition using P. bergheii, P. yoelii and P. falciparum model: pre and post inhibition of the cytokines. Action of NGMML-arginine as competitive inhibitor of NO synthesis from L-arginine. Measure of NO production by infected hepatocytes. Measure of protein synthesis. Measure of cytokines and co-factors and production of NO during the immunization with irradiated-sporozoites (P. yoelii model) in the immune liver and spleen.

Progress: We have demonstrated using the in vitro P. berghei model the involvement of NO, a toxic effector, in the IFNg killing: hepatocytes produce NO under IFNg treatment and after having been infected by the parasite. No other cytokine, so far, is involved for the NO production: the parasites play the role of second activator. Preliminary data on human model (P. falciparum/ human hepatocytes) indicate the same phenomen. Immune organs (liver and spleen) are involved in processing.

Plan: Determination of the mechanism of killing of infected hepatocytes by immunocompetent cells: direct killing by contact or by release of cytokines, involvement of NO.

Publication:
1. Mellouk S, Green S, Nacy C and Hoffman SL . IFN-g development of Plasmodium berghei exoerythrocytic stages in hepatocytes by an L-arginine-dependent effector mechanism. J Immunol, 146: 3971-3976, 1991.

Attacking the infected hepatocyte (primary)

Project Title: What happens inside the liver during irradiated sporozoite immunization?
Funding: 6.1
Principal Investigator: Sylvie Mellouk, PhD
Co-Investigators: Benjamin Wizel, PhD
Patricia de la Vega, BSc

Objective : Isolation, analysis and role of cell populations infiltrating the liver during the irradiated-sporozoite immunization.

Methods: Isolation of cells from liver and spleen, analysis by IFA , FACS, using Mabs available (T-cells [CD4, CD8], B cells, NK cells). Direct cytotoxic killing of infected hepatocytes. Boost in vitro with antigens (CSP, SSP2) or EE stages and 51Cr cytotoxic assays or killing of infected hepatocytes. Transfer in vivo. Clonage.

Progress: Preliminary experiments have been done in the attempt to isolate monuclear cells from the liver without perturbing their immunogical markers and functions. By using non-proteolytic treatment we were able to collect 2 to 3 x 106 cells per organ. The analysis of the phenotype of those cells by FACS shows that the majority are lymphocytes but also demonstrates the presence of myelomonocytic cells. The phenotype of the cells has to be more closely analysed via different markers. In a single experiment, these cells were able to kill 74% of infected hepatocytes.

Abstracts: N/A

Publications: N/A

Attacking sporozoites,
Attacking infected hepatocytes (primary)

Project Title: Recombinant pseudorabies virus carrying a malaria gene: herpesvirus as a new live viral vector for inducing T and B cell immunity

Funding: 6.2

Principal investigator: Martha Sedegah, PhD

Co-Investigators: Christina H Chang, PhD, SYNTRO, CA
Walter R Weiss, CDR, MC, USNR
Sylvie Mellouck, PhD
Mark D Cochran, PhD, SYNTRO Corp, CA
Ricdard A. Houghten, PhD, Torrey Pines Institute for Molecular Studies, CA.
Stephen L Hoffman, CDR, MC, USNR

Objectives: To produce a vaccine that would induce T and B cell immunity against the *P. yoelii* circumsporozoite protein.

Methods: An attenuated pseudorabies virus (PRV) containing a gene encoding the CS protein of *P. yoelii* was used to immunize BALB/c mice. Immune response as measured by antibodies(IFA and ELISA) and CTL responses were measured. Mice were also challenged with infective sporozoites to look for protective immunity. Furthermore, the ability of the CTL produced to eliminate *P. yoelii* - infected hepatocytes from in vitro culture was tested.

Progress: The immunized mice developed high levels of antibodies to sporozoites and CTL against the CTL epitope on *P. yoelii* CS protein. Furthermore, these CTL eliminated *P. yoelii*-infected hepatocytes from *in vitro* culture, indicating that they recognize this peptide on the surface of infected hepatocytes. However, all nine mice that were challenged with 200 sporozoites developed a blood-stage malaria infection. We attribute this lack of protection to the great difficulty of inducing sterile immunity against this highly infectious parasite *P. yoelii*.

Abstracts:

1. Sedegah M, Chiang CH, Weiss WR, Mellouk S, Cochran MD, Houghten RA, Beaudoin RL, Smith D, and Hoffman SL. Immunization with a transformed pseudorabies virus induces cytotoxic T lymphocytes that recognize a 16 amino acid peptide from the *P. yoelii* circumsporozoite protein on the surface of infected hepatocytes. The 39th Annual Meeting, Amer Soc Trop Med Hyg, New Orleans, LA, Nov 1990.

Publications:

1. Sedegah M, Chiang CH, Weiss WR, Mellouk S, Cochran MD, Houghten RA, Beaudoin RL, Smith D, and Hoffman SL. Recombinant pseudorabies virus carrying a malaria gene: herpesvirus as a new live viral vector for inducing T and B cell immunity, Vaccines, in press.

Attacking infected hepatocytes (primary)

Project Title: Naturally acquired CD8+ cytotoxic T lymphocytes against the *Plasmodium falciparum* circumsporozoite protein

Funding: 6.2

Investigators:
Martha Sedegah, PhD
B. Kim Lee Sim, PhD,WRAIR
James Sherwood, USAMRU
Carl Mason, PhD, WRAIR
Tom Nutman, PhD, NIH
Anita Malik, PhD
Clifford Roberts, LTC VC USA, USAMRU
Armeand Johnson, PhD, Georgetown Univ. Wash. D.C.
Jessica Ochola, MD, KEMRI, Kenya
Davey Koech, MD, KEMRI, Kenya
Ben Ware, MD, KEMRI, Kenya
Stephen L. Hoffman. CDR. MC, USNR.

Objectives: The studies were undertaken to determine if individuals from Kenya, previously shown to be relatively resistant to infection to *P. falciparum*, and whose lymphocytes proliferate after stimulation with peptides 361-380, 368-390, or 371-390 of the *P. falciparum* CS protein, had circulating CD8+ cytolytic T lymphocytes against epitopes within amino acid residues 368-390.

Methods: PBMNC's from the volunteers were stimulated in vitro for 6 days in the presence of peptide Pf 7G8 368-390 and used as effectors in a standard chromium release assay using EBV transformed B cells as targets.

Progress: In four of the 11 individuals there was peptide specific, genetically restricted CTL activity. In all 4 individuals, this activity was unaffected by depletion of CD4+ T cells. In 3 volunteers, the activity was eliminated or reduced by CD8+ T cells depletion. None of the four individuals shown to have cytolytic activity had asexual *P. falciparum* parasitemia, while four of the 7 individuals without cytolytic activity had asexual parasites.

Plans: Follow up studies are planned to identify many more CTL positive volunteers using much shorter peptides which could give optimal responses. We would also establish clones which would be used to study genetic restriction to CTL epitopes and also to determine if CTL responders produce CTL against other epitopes on the *P. falciparum* CS protein. Finally, considerable work will be required to determine the significance of these anti-CS protein CTL and CTL against other pre-erythrocytic proteins, including the sporozoite surface protein 2 equivalent in *P. falciparum*, and to develop subunit vaccines that induce functionally active CTL directed against these proteins. In addition a collaborative CTL network is being established with investigators in Cali, Colombia (M. and S.

Herrera), Accra, Ghana (F. Nkrumah and M. Sedegah), Kisumu and Nairobi, Kenya (see above), Bangkok, Thailand (S. Khusmith), and Jakarta, Indonesia (T. Ritchie).

Abstracts:

1. Sedegah M, Sim BKL, Sherwood J, Mason C, Nutman T, Malik A, Roberts C, Johnson A, Ochola J, Koech D, Ware B, Hoffman SL. Human cytotoxic T lymphocytes against the *Plasmodium falciparum* circumsporozoite protein after natural exposure to malaria. 40th Annual Meeting, Ameri Soc Trop Med Hyg, Boston, MA. Dec, 1991.

Publication:

1. Sedegah M, Sim BKL, Sherwood J,Mason C, Nutman T, Malik A, Roberts C, Johnson A, Ochola J, Koech D, Ware B, Hoffman SL. Naturally Acquired CD8+Cytotoxic T Lmphocytes Against the *Plasmodium falciparum* Circumsporozoite Protein. J Immunol, in press.

Attacking infected hepatocytes (primary)

Project Title: Identification of CTL against the P. falciparum CS protein in volunteers immunized with a live oral S. typhi strain carrying the gene for the P. falciparum CS protein.

Funding: 6.3

Principal Investigator: Anita Malik, PhD
Co-Investigators: Martha Sedegah, PhD
Ben Wizel, PhD
Marcelo Stzein, MD
Carol Tacket, MD

Objectives: To determine if humans immunized with a live oral S. typhi strain carrying the gene for the P. falciparum CS protein produce CTL against the P. falciparum CS protein.

Methods: Twelve volunteers will be immunized with two formulations of a live oral S. typhi strain carrying the gene for the P. falciparum CS protein, and 3 and 6 weeks later will be tested for CTL. Their B cells will be transformed with EB virus, their cells will be stimulated in vitro with a synthetic peptide from the CS protein or with a recombinant vaccinia virus carrying the P. falciparum CS protein, and cytolytic activity will be tested against their own B cells transfected with the gene encoding the CS protein or with peptide pulsed targets.

Progress: Twelve volunteer have been immunized.

Plans: If CTL are identified, the volunteers will have leukopheresis and attempts will be made to clone their T cells.

Abstract: N/A

Publication: N/A

Attacking infected hepatocytes (primary)

Project Title: Stable chromosomal integration of sporozoite stage malaria antigens (CS protein and PFSSP-2) into EBV transformed B Cells of human volunteers.

Funding: 6.2/6.3

Principal Investigator: Anita Malik, PhD

Co-Investigators: Sanjai Kumar, PhD, NIAID NIH
Craig Mullen, MD, PhD, NCI NIH
William O. Rogers LCDR, MC, USNR

Objectives: Generation of stable transfectants as the source of antigen presenting cells or stimulator cells for CD8+ and CD4+ T cell lines and clones from human colunteers immunized with irradiated sporozoites or subunt CS protein formulation.

Methods: For the stable transfection P.falciprum CS or SSP-2 genes in the EBV transformed cells of human volunteers, these genes were cloned in retoviral vector pLXSN at the ECORI restriction site. Tissue cell lines PA317 and GPE86 were transfected with plasmid DNA. Retroviral producing these lines then checked for protein expression (PfCS/SSP-2) by western blotting. Each volunteers EBV transformed B cells were then transfected with PfCS/SSP-2 genes by coculturing them with virions producing packaging cell lines(PA 317-GPE86). Transfected cells were then selected in G418 selective medium and checked for protein expression by western and northern blotting.

Progress: Generation of retroviaral vector producing cell lines: Genes coding for PfCSP and PfSSP-2 were cloned into retroviral vector pLXSN at the ECORI restriction site. Recombinant retroviral producing cell lines were made by transfection of ecotopic retrovirus packaging GPE86 cell line and amphitropic cell line PA317. G418 resistant colonies were grown and then assayed for virus titre. Retroviral mediated transfer of PfCSP and PfSSP-2 into EBV transformed B cells of human volunteers was done by coculturing the virus producing packaging cell lines with each volunteers B cells individually. Transfected cells were then grown in G418 - selective medium.

Plans: Expression of PfSSP2 and PfCSP in the stable transfectents will be checked by western and northern blotting and they will be used to generate T-cells lines and clones from volunteers immunized with sporozoites.

Abstract: N/A

Publication: N/A

Attacking infected hepatocytes

Project Title: T-cell response against PfSSP-2 in human volunteers immunized with irradiated sporozoites of *P. falciparum*
Funding: 6.3
Principal Investigator: Anita Malik, PhD
Co-Investigators: William O. Rogers, LCDR, MC, USNR

Objective: PySSP2 is an important protective antigen in the murine model. PfSSP2 is a homolog of PySSP2 in *P. falciparum*. We plan to determine the T-cell responsivieness to PfSSP2 in human volunteers immunized with irradiated sporozoites.

Methods: A dra I/Hind III fragment of PfSSP2 was cloned into the expression vector pGEMEX and resultant plasmid was introduced into *E.coli* . Insoluble protein suspensions from *E. coli* HMS174(DE3)/pLysS bearing either pPfSSP2.GXD3 or pGEMEX were subjected to preparative SDS-PAGE, transferred to nitrocellulose paper, and stained with 0.01% amido black. Bands corresponding to the PfSSP2 fusion protein, to the T7 gene 10 fusion partner alone and to insoluble *E. coli* proteins were excised and processed to nitrocellulose micro particles. Nitrocellulose particles containing the above proteins were used in standard T cell proliferation assays.

Progress: Human T cell responses to PfSSP2: Peripheral blood leukocytes from 3 of 5 volunteers examined proliferated in response to the PfSSP2 fusion protein. Immune cells, but not preimmune cells, proliferated in response to stimulation with the PfSSP2 fusion protein, but not to stimulation with the fusion partner alone, or with nitrocellulose beads alone.The evidence that T cells from sporozoite immunized volunteers in vitro proliferate with PfSSP2 suggests that this protein is expressed in pre-erythrocytic stages

Abstract: N/A.

Publications:
1. Rogers WO, Malik A, Mellouk S, Nakamura K, Gordon DM, Rogers MD and Hoffman SL. *Plasmodium falciparum* sporozoite surface protein 2, a sporozoite protein distinct from the circumsporozoite protein. Submitted for publication.

Attacking infected hepatocytes (primary)

Project Title: Induction of CTL response against *P. falciparum* CS Protein
Funding: 6.3
Principal Investigator: Anita Malik, PhD
Co-Investigators: Mitchell Gross, PhD, SmithKline Beecham
Terry Ulrich, PhD, Ribi ImmunoChem Inc

Objectives: Induction of CTL response by immunization with soluble protein.

Methods: B10.BR mice were immunized iv, ip or im routes with a recombinant fusion protein that includes entire *P. falciparum* CS protein minus 164 amino acids from the central repeat region linked to 81 amino acids from a non-structural protein of influenza A (RLF), with or without adjuvant DETOX (MPL/CWS and squalane) spleen cells from these mice were stimulated invitro with PfCS gene transfected L cells and then checked for their cytolytic activity in CTL assay.

Progress: To determine if we can induce CTL against the *P. falciparum* CS protein by immunization with soluble recombinant protein, B10.BR mice were immunized iv, ip or im with a recombinant *P. falciparum* CS protein called RLF, mixed with the adjuvant DETOX™. Two weeks after the last dose, spleen cells from mice immunized iv, but not ip or im, had peptide specific, MHC restricted, CD8+ T cell-dependent cytolytic activity against peptide 368-390 from the 7G8 *P. falciparum* CS protein. To determine if the adjuvant was required for induction of the cytolytic activity, mice were immunized with RLF without adjuvant, and similar cytolytic activity was demonstrated. The finding that we could induce CTL by administration of soluble protein without adjuvant markedly broadens the possibilities for vaccinologists working to develop methods of inducing CTL in humans.

Plans: We will try immunize mice with CTL epitope Pf CS-(368-390) alone and see if we can induce CTL just by immunizing peptide alone.

Abstract:
1. Malik A, Gross M, Ulrich T and Hoffman SL. Induction of cytotoxic T lymphocytes against the *Plasmodium falciparum* CS protein by immunization with soluble protein without adjuvant. 40th Annual meeting, Amer Soc Trop Med Hyg. Boston, MA, Dec 1-5, 1991.

Publication:
1. Malik A, Gross M, Ulrich T and Hoffman SL. Induction of cytotoxic T lymphocytes against the *Plasmodium falciparum* circumsporozoite protein by immunization with soluble recombinant protein without adjuvant. Submitted for publication.

Attacking infected hepatocytes (primary)

Project Title: Human cytotoxic T -lymphocytes against the *Plasmodium falciparum* CS protein.
Funding: 6.3
Principal Investigator: Anita Malik, PhD
Co-Investigators: Dan Gordon, LTC, MC, USA, WRAIR
Richard Houghten, PhD, Torrey Pines Institute of Molecular Studies

Objectives: CTL against the CS protein of malaria sporozoites protects against malaria in rodents. Accordingly there are efforts to develop vaccine that induce CTL against *Plasmodium falciparum.* Our goal is detect CTL response in human volunteers immunized with irradiated sporozoites or CS subunit vaccines and map the CTL epitope.

Methods: PBMC from human volunteers were stimulated in vitro with a recombinant vaccinia virus containing PfCS gene or peptide Pf CS 368-390 and were then used as effectors in CTL assays,using each volunteer's EBV transformed B cells as target cells. Target cells were either coated with peptide 368-390 or transiently transfected with *P. falciparum* CS gene.

Progress: PBMC from three of four human volunteers immunized with irradiated *P. falciparum* sporozoites were stimulated in vitro with a recombinant vaccinia virus expressing the *P. falciparum* CS protein or a peptide including only amino acids 368-390 (CS-368-390) of the *P. falciparum* CS protein. These effectors lysed autologus EBV transormed B cells transfected with the *P. falciparum* CS gene or incubated with CS-(368-390). The CTL activity was antigen specific, genetically restricted and dependent on CD8+ T cells. In one volunteer, seven peptides reflecting amino acids 311-400 were tested and CTL activity was associated with CS-(368-390) peptide. Development of an assay for studying human CTL against the CS and other malaria proteins and methods for constructing target cells by direct gene transfection provide a foundation for studying the role of CTL in protection.

Plans: We plan to make CTL clones against Pf CS-(368-390) and map the minimal CTL epitope using truncated peptides from region 368-390. We will continue to use this assay to determine the CTL response of humans immunized with malaria vaccines.

Abstracts:
1. Malik A, Egan JE and Hoffman SL. Cytotoxic T lymphocytes against the *Plasmodium falciparum* circumsporozoite protein. FASEB Journal, 75th Annual Meeting, Atlanta, GA, April 91.

Publications:

Malik A, Egan JE, Houghten RA, Sadoff JC and Hoffman SL. Human cytotoxic T lymphocytes against the *Plasmodium falciparum* circumsporozoite protein. Proc Natl Acad Sci USA 88:3300-3304, 1991.

Attacking infected hepatocytes (primary)

Project Title: Characterization of CD8+ clones against *P. yoelii* SSP2 with different *in vivo* cytotoxic activities

Funding: 6.2

Principal Investigator: Benjamin Wizel, PhD
Co-investigators: Sylvie Mellouk, PhD
Srisin Khusmith, PhD
Richard Houghten, PhD, Torrey Pines Institute for Molecular Studies

Objective: To identify, determine and characterize the basis for the heterogeneity displayed by three *P. yoelii* SSP2 CTL clones with regard to the strikingly different levels of protection these clones provide in adoptive transfer experiments.

Methods: The 3 PySSP2 clones, TSLB7, TSL10.21 and TSL14.18, recognize the same peptides in a genetically restricted fashion upon their *in vitro* analysis in CTL assays using MHC matched peptide-pulsed cells and transfectants expressing a 1.5 kb fragment of the PySSP2. Adoptive transfer experiments as well as *in vitro* killing of Plasmodium-infected hepatocytes demonstrated the presence of protective and non-protective CTL clones. To elucidate the mechanism(s) by which apparently identical CTL clones against PySSP2 exhibit marked differences in their *in vivo* cytotoxic activities, studies will be undertaken to: 1) characterize the fine specificity (to define the minimal epitope) using truncated peptides to pulse MHC-matched targets in classical CTL assays, 2) investigate the pattern of lymphokine production by measurement, amongst others, of IFN-g and TNF on supernatants of specifically stimulated CTL clones, 3) analize the phenotype of the CTL clones with regard to the expression of adhesion molecules and homing ligands by flow cytometry, and 4) to determine tissue migration specificities using adoptive transfer of radiolabeled CTL clones and subsequent histological analysis of tissue sections.

Progress: new start

Plans: Once differences of protective and non-protective CTL clones have been elucidated, *in viiro* killing of Plasmodium-infected hepatocytes will be used to dissect precisely the mechanisms by which protective CTL clone mediates its biological function. In addition, cultures of *P. yoelii* infected hepatocytes will be used as a source to purify MHC molecules from which bound peptides will be eluted and sequenced.

Abstracts: N/A

Publications:

1. Khusmith S, Mellouk S, Sedegah M, Houghten R and Hoffman SL. Complete

protection against *Plasmodium yoelii* by adoptive transfer of a CD8+ cytotoxic T cell clone recognizing Sporozoite Surface Protein 2. Submitted for publication.

Attacking infected hepatocytes (primary)

Project Title: Characterization of murine T cell immune responses to *P. falciparum* sporozoite surface protein 2 (PfSSP2)
Funding: 6.2
Principal Investigator: Benjamin Wizel, PhD
Co-investigators: Richard Houghten, PhD, Torrey Pines Insitute of Molecular Studies

Objective: To identify and characterize T cell-mediated immune responses (T helper and T cytotoxic) against PfSSP2 in mice.

Methods: Mice will be immunized with *P. falciparum* sporozoites, live vectors such as vaccinia, Salmonella sp. and BCG transformed with the gene encoding PfSSP2, synthetic peptides derived from PfSSP2 sequence and MHC-compatible cells transfected with the gene encoding SSP2. Murine immune spleen cells will then be stimulated *in vitro* with MHC-matched PfSSP2 gene transfected cells or with peptide-pulsed MHC-histocompatiable cells to determine their capacity to lyse the same transfectants or similar cells incubated with peptides derived from PfSSP2 sequence. Having established the presence of such cytotoxic T lymphocytes, CTL clones will be derived to characterize their fine specificity for the most critical CTL epitopes using truncated peptides to pulse appropriate targets. Transfected cells containing smaller fragments of the PfSSP2 gene will be constructed to provide additional information in mapping the minimal CTL epitope. In addition, recombinant PfSSP2 and synthetic peptides derived from its sequence will be used to identify and characterize epitopes recognized by T helper cells.

Progress: New start

Plans: As described in methods.

Abstracts: N/A

Publications: N/A

Attacking sporozoites.
Attacking infected hepatoctyes (primary).
Attacking blood stage parasites.

Project Title: A study correlating T cell reactivity to the CS protein with resistance to falciparum malaria infection in Western Kenya.

Funding: 6.1
Principal Investigator: Walter Weiss, CDR, MC, USNR, NMRIDET-KENYA
Co-Investigator: Kim Lee Sim, Ph.D., WRAIR
Stephen L. Hoffman, CDR, MC, USNR

Objective: Correlate T lymphocyte reactivity to the CS protein of *P. falciparum* with resistance to malaria infection in heavily exposed population in Western Kenya. This information on malaria immunity will be used to guide malaria vaccine development.

Methods: Adult men will be recruited from villages in western Kenya where malaria is highly endemic. They will be cured of pre-existing malaria infections using quinine and doxycycline, and then followed by weekly malaria smears during the 12 week rainy season to see when and if they develop malarial parasite infections. At the end of this time, lymphocytes will be taken from men who did not get malaria and a comparison group who became ill in the first quarter of the study. In vitro studies using these cells will include cytotoxic activity, and gamma-interferon and IL-4 release when stimulated by the CS protein. In addition, two other malaria antigens, the 70 KD heatshock protein, and RESA will be tested for comparison with the CS. Any activity will be localized to CD4+ or CD8+ T cell subsets.

In the second part to the study, the importance of T cell escape mutants in malaria will be evaluated. Subjects with documented T cell responses will be followed for malaria through a second rainy season. If they get malaria, blood will be taken and parasite DNA for the CS protein sequenced to see if the infecting parasite has a variant sequence. If such variations are found, synthetic peptides corresponding to these variants will be tested to see if the subject lacks T cell responses to these mutations.

Progress: Three previous DOD sponsored studies in Kenya have shown that 1) T cell proliferation to CS epitopes is correlated with resistence to malaria infection, and 2) cytotoxic T cell activity is demonstrable in subjects from this area. These preliminary studies provide the groundwork for an indepth analysis of T cell immunity and resistance to malaria infection. As of May 92, our protocol is under final stages of approval by the Kenya Medical Research Institute and WRAIR scientific and ethical review committees. Volunteers have been selected and we anticipate beginning screening for malaria incidence in May 92. A field laboratory has been established in western Kenya where pilot in vitro studies are in progress.

Publications:

1. Hoffman SL, Oster CN, Mason C, Beier JC, Sherwood JA, Ballou WR, Mugambi M and Chulay JD. Human Lymphocyte Proliferative response to a sporozoite T cell epitope correlates with resistance to *falciparum* malaria. J Immunol 142:1299-1303, 1989
2. C. Mason et al. manuscript in preparation.
3. M. Sedegah et al. Naturally acquired CD8+ Cytotoxic T lymphocytes against the *Plasmodium falciparum* circumsporozoite protein. Submitted for publication.

Attacking infected hepatocytes (primary)
Attacking blood stage parasites

Project Title: Identification and characterization of the *P. yoelii* liver stage antigen
Funding: 6.1/6.2
Principal Investigator: Yupin Charoenvit PhD
Co-Investigators: Martha Sedegah PhD
Sylvie Mellouk PhD
Patricia de la Vega, BSc
Stephen L. Hoffman, CDR, MC, USNR

Objective: To identify, characterize and produce liver stage antigen(s) that may be important for malaria vaccine development. In this study we are using *P. yoelii,* a murine malaria as an animal model system. The first goal is to produce a series of anti-liver stage monoclonal antibodies (Mabs). After being characterized, the Mabs are used to identify and characterize liver stage antigens produced by *P. yoelii* infected hepatocyte. The second goal is to use these Mabs to identify liver stage antigens produced by recombinant DNA technology. The liver stage antigen of interest will be used in active immunization studies.

Method: To produce anti-liver stage Mabs, we immunized mice with 44 hr-*P. yoelii* liver stage schizonts. Spleen cells from these mice were fused with a mouse myeloma cell line using conventional fusion methods. The hybrid lines secreting antibodies against liver stage parasites were cloned and the anti-liver stage Mabs produced by these clones were characterized. In the studies to produce liver stage antigen by recombinant DNA technology, genomic DNA library was produced from *P. yoelii* blood stage parasites, cloned into lambda gt 11 bacteriophage vectors. The recombinant DNA clones were then used to infect the *E.coli* hosts. The recombinant clones expressing liver stage antigens were screened in the ELISA by using liver stage Mabs.

Progress: A series of Mabs directed against *P. yoelii* liver stage parasites has been produced. These Mabs react in the immuno-fluorescent test (IFAT) with liver stage parasites in both *in vitro* and *in vivo* infected hepatocyte. NYLS3 (Navy yoelii liver stage 3) has been further characterized. This Mab is an IgG1 isotype, recognizes liver and blood stage parasites, but not sporozoites by the IFAT. NYLS3 also recognizes a 17 Kd protein in extracts of blood stage parasites by SDS/PAGE immunoblotting analysis. It dose not recognize *P. berghei* liver stage parasites. When this Mab is added to hepatocyte cultures at 24 hr after sporozoite invasion, it inhibits schizont development by 45%. The inhibitory effect reaches 80% when mineral oil activated macrophages are also added to the cultures. NYLS3 identified a *P. yoelii* gene fragment encoding a portion of the protein, LISA3. Preliminary sequence data indicates that LISA3 is homologous to the *P. falciparum* protein, Pf. Exp-1.

Plans: The gene encoding LISA3 antigen will be transfected into P815

mastocytoma (eucaryotic) cells and the transfectant expressing this antigen will be used in active immunization studies.

Abstracts:

1. Charoenvit Y, Mellouk S, Sedegah M, Leef MF, de la Vega P, and Hoffman SL. Characterization of an inhibitory monoclonal antibody against *Plasmodium yoelii* liver stage parasites. 40th Annual Meeting, Amer Soc Trop Med Hyg, Boston, MA, Dec 91.

Publication:

1. Charoenvit et al. Characterization of an inhibitory monoclonal antibody against liver stage of *P. yoelii*. manuscript in preparation.

Attacking sporozoites (primary).
Attacking infected hepatoctyes.
Attacking merozoites (primary).

Project Title: Additive or synergistic protection after passive transfer of anti-sporozoite and anti-erythrocyte stage malaria antibodies: rational for the development of a multicomponent malaria vaccine

Funding: 6.1/6.2

Principal Investigator: Martha Sedegah, PhD
Co-Investigators: Carole A Long, PhD, Hahnemann University, Philadelphia.
Yupin Charoenvit, PhD
Stephen L. Hoffman, CDR, MC, USN

Objective: To form a rational for the development of a multicomponent malaria vaccine by combining Mabs to different stages of the malaria parasite in a passive transfer study.

Methods: BALB/C mice are passively transferred with low dose of the protective anti-CS protein Mab NYS1 and the mice are challenged 30 mins later with 200 infective *P. yoelii* sporozoites. After a further 24 hours, the mice receive 2mg of a second mab (mab 302) against the major merozoite surface protein 1 (MSP-1) of *P. yoelii*.

Progress: The data so far demonstrate synergism in regard to protection against blood stage parasites using combined monoclonal antibodies directed against the sporozoite and erythrocytic stages of the rodent malaria, *P. yoelii*. Passive transfer of low dose of the anti-CS protein Mab NYS1 protected a percentage of mice against sporozoite challenge and transfer of 2mg Mab 302, a Mab against the 230 kD, P. yoelii MSP-1 leads to low grade parasitemia in all reciepient mice upon challenge with sporozoites. However when the two mabs were both administered to mice, most of the mice did not develop detectable parasitemia. In a total of 3 experiments, 15 out of 19 mice had no detectable parasitaemia compared to only 6 out of 20 in the group that received the low dose NYS1 alone. All of the 20 mice that received mab 302 alone developed low grade parasitaemia.This absence of detectable parasitemia was dependent on the dose of sporozoite used for the challenge as well as the dose of the anti-MSP-1 Mab.

Plans: We are in the process of preparing large quantities of NYS1 and 302 so as to more completely characterize this protection and determine the best regimen. In addition since this Mab recognizes a conformational epitope in the C-terminus of PyMSP-1, a portion of the gene encoding PyMSP-1 is being transfected into mammalian cells (P815) for eventual use as a vaccine that will be combined with vaccines designed to induce antibodies to the PyCSP. Likewise this region has also been expressed by Baculovirus, and this recombinant protein will be used in the immunization studies.

Publications: Manuscript on the passive transfer studies in preparation.

Attacking sporozoites.
Attacking infected hepatoctyes.
Attacking merozoites (primary).

Project Title: Correlations between cellular and humoral immune responses to defined *Plasmodium falciparum* antigens and susceptibility to malaria infection.

Funding: 6.1

Principal Investigator: Ronald L. Anthony, Ph.D., NAMRU-2
Co-Investigator: Michael J. Bangs, LCDR, MSC, USN, NAMRU-2
J. Kevin Baird, LCDR, MSC, USN, NAMRU-2

Objective: To demonstrate a positive correlation between the humoral/cellular immune response to defined *P. falciparum* antigens (CSP, LSA, RESA, MSA-1 and MSA-2) and resistance and/or susceptibility to malaria infection in children and adults living under conditions of intense stable transmission.

Methods: This longitudinal study is focused upon defining the host-vestor-parasite interelationships which regulate the prersistence/transience of T-cell/B-cell recognition of specific *Plasmodium falciparum* antigens. The study is being conducted in Oksibil, a highland valley in Irian Jaya, Indonesia where the 2000 members of the Ngalum tribe are living under conditions of intense stable transmission. Malariometric surveys are conducted quarterly and blood specimens for lymphocyts responsiveness, phenotyping, antibody assays, monokine levels and *P. falciparum* culture are collected.

Progress: Over the past 2.5 years, the Oksibil study site has been defined demographically, epidemiologically, entomologically, parasitologically and immunologically. Malaria prevalence rates years of age, 35% for the 5-9 year olds, 28% for the 10-14 year-old groups and 16% for adults >15 years. The spleen rate for the <5 year olds in 96% with an average enlarged spleen score of 2.32. *P. falciparum* accounts for 55% of the infections in one village; 4 cases were diagnowed as *P. ovale*. Approximately 10% of the adults, although having lived under conditions of stable transmission for years, fail to develop naturally acquired immunity or clinical tolerance. Sixty-five percent of the adults commonly have infections without clinical symptoms; 25% have shown no parasitologic evidence of infection after two years of close monitoring. Man biting rates have been established at 0.6 infective bites/person/night.

A bank of >1000 Oksibil sera contains serial specimens from highly susceptible children, susceptible adults and resistant adults. All age groups are represented. In many cases, sera are available from a single individual a few days before parasitologic evidence of infection, during infection and a few days to a month after parasitemia had cleared. High titers of serum antibodies which recognize >35 peptides of *P. falciparum* erythrocytic stage antigens do not prevent reinfection with *P. falciparum*. Preliminary results do suggest that some clinically tolerant adults show a strong lymphoproliferative response to Fab anti-

idiotypes prepared from serum with high titers of anti-*P. falciparum* erythrocytic stage antibodies.

Publications:

1. Anthony RL, Bangs MJ, Hamzah N, Basri H, Purnomo and Subianto B. Heightened transmission of stable malaria in an isolated population in the highlands of Irian Jaya, Indonesia. Amer J Trop Med Hyg, in press.

2. Anthony, RL, Bangs MJ, Anthony JM, and Purnomo. On-site diagnosis of *Plasmodium falciparum, P. vivax*, and *P. malariae* by using the quantitative buffy coat system. J Parasitol, in press.

3. Bangs MJ, Purnomo, and Anthony RL. 1992. A focus of *Plasmodium ovale* in the highlands of Irian Jaya. Ann Trop Med Hyg, in press.

Treatment

Project Title: The role of intracranial hypertension in the morbidity and mortality of cerebral malaria.
Funding: 6.3
Principal Investigator: James H. Bower, LT, MC, USNR
Co-Investigator: Stephen L. Hoffman, CDR, MC, USNR

Objective: To evaluate the prevalence of intracranial hypertension in patients with cerebral malaria, and to research possible therapeutic interventions to control the intracranial pressure.

Methods: A retrospective chart review was performed on 128 patients see with cerebral malaria from October, 1982 through March, 1985 at the Provincia. Hospital in Jayapura, Irian Jaya, Indonesia. Charts were reviewed for clinical status, CSF results, opening pressure measurements on lumbar puncture, parasitemia levels and outcome. A literature search was also performed to collate the data on opening pressure measurements noted in other studies to determine whether intracranial hypertension is a consistent finding in cerebral malaria.

Progress: 12/12 (100%) of children (<14 yrs.) were found to have an increased ICP on opening pressure measurements, while 6/12 (50%) of adults were found to have an increased ICP. 17/24
of those patients had a normal CSF (<6 WBC), and 7/24 had a moderate pleocytosis (6-50 WBC). Of the 17 patients with a normal CSF cell count, 9/9 (100%) of children had an elevated pressure, while 4/8 (50%) adults had an increased ICP. From the review of studies noting opening pressure measurements, it was found that the percentage of adult patients with intracranial hypertension varied from 2-44%, while the percentage of children varied from 50-100%. It is clear that intracranial hypertension plays an important role in cerebral malaria in children, and may also play an important role in at least some adults. Possible therapies to reduce ICP in these patients include head elevation, hyperventilation and Mannitol treatment.

Plans: This review was important to determine the role of intracranial hypertension in cerebral malaria, so as to affect future treatment options in cerebral malaria patients. A prospective study using different interventions is being considered.

Publications: Hoffman SL, Rustama D, Punjabi NH, Surampaet B, Sanjaya B, Dimpudus AJ, McKee KT, Paleologo FP, Campbell JR, Marwoto H and Laughlin L. High-dose dexamethasone in quinine-treated patients with cerebral malaria: a double-blind, placebo-controlled trial. J Infect Dis, 158: 325-331, 1988.

Treatment

Project Title: A field trial of primaquine as a prophylactic drug against *flaciparum* malaria

Funding: 6.3
Principal Investigator: Walter Weiss, LCDR, MC, USNR, NMRIDET-KENYA
Co-Investigators: Stephen L. Hoffman, CAPT, MC, NMRI

Objective: To test the efficacy of prophylactic primaquine in preventing new cases of falciparum malaria in an endemic area.

Methods: Eighty school children living in an area hyper-endemic for falciparum malaria will be medically screened, then cured of pre-existing malaria by quinine and doxycycline therapy. They will then be randomized to receive either primaquine (15mg) or placebo on Monday, Wednesday, and Friday during 12 weeks of the rainy season. A malaria blood film will be evaluated weekly on each child and will be evaluated for malaria should they fall ill for any reason. This study should be able to show whether this regime has 80% or greater efficacy in preventing *falciparum* malaria (p=0.05, beta=0.10) given an expected incidence of 50% or greater in the control group.

Progress: As of May 92, approvals for this study have been received from the Scientific and Ethical Review committees of the Kenya Medical Research Institute and the Walter Reed Army Institute of Research. Children have been enrolled and medically screened and we expect to begin drug therapy in May 92.

Plans: If this primaquine regimen shows any efficacy, we will test other primaquine dosage schedules, and expand the study population to include adults. We will also be in a position to test new drugs being developed at WRAIR which have the same schizonticidal action as primaquine, but more favorable pharmacokinetics.

Treatment

Project Title: Characterization of the pathogenesis of severe malaria and development of treatment strategies
Funding: 6.3
Principal Investigator: Thomas L. Richie, LCDR, MC, USNR, NAMRU-2
Co-Investigators: Stephen L. Hoffman, CDR, MC, USNR
J. Kevin Baird, LCDR, MSC, USN, NAMRU-2

Objectives: 1) To characterize the role of elevated levels of lymphokines and oxygen radicals, and erythrocyte rosetteing in the pathogenesis of severe malaria. 2) To develop alternative treatments for severe malaria based on these findings, such as anti-TNF monoclonal antibodies, pentoxifylline and oxygen radical scavengers. 3) To identify risk factors or protective factors for developing severe malaria, such as HLA type and hemoglobin type.

Methods: For identification of pathophysiological mechanisms and risk/protective factors in severe malaria, patients hospitalized with cerebral malaria will be compared with matched hospitalized controls in a prospective study. Positive or negative correlations between measured variables and cerebral involvement will be sought. For testing new treatments, cerebral malaria patients will be enrolled in a randomized, double-blind, placebo-controlled trail.

Progress: A study population has been identified in Irian Jaya, Indonesia that is appropriate for investigating the pathogenesis of severe malaria. The Jayapura General Hospital, which admits approximately 80 cerebral malaria patients per year, has agreed to collaborate with NAMRU-2 in the first phase of this project: the characterization of lymphokine and nitric oxide levels, erythrocyte rosetting, and HLA and hemoglobin types in patients with cerebral malaria. Permission has also been granted to study a second population of malaria patients, admitted to Manado General Hospital, Sulawesi, Indonesia. Depending upon the results of the first phase of this project, health officials at both hospitals have agreed in principle to support the second phase: a trial of interventional agents designed to reverse pathogenic mechanisms, such as anti-tumor necrosis factor monoclonal antibodies. It is hoped that such new therapies will reduce the 20% mortality currently associated with cerebral malaria.

Plans: Complete the first phase of the project (pathophysiology) by early 1993. The second phase hinges upon the outcome of the first; we plan tentatively to begin mid-1993.

Publications:
1. Hoffman SL, Rustama D, Punjabi NH, Surampaet B, Sanjaya B, Dimpudus AJ, McKee KT, Paleologo FP, Campbell JR, Marwoto H, Laughlin L. High-dose dexamethasone in quinine-treated patients with cerebral malaria: a double-blind, placebo-controlled trial. J Infect Dis, 158:325-331, 1988.

Treatment

Project Title: Chloroquine-Resistant *Plasmodium vivax* in Indonesia.
Funding: 6.2
Principal Investigator: J. Kevin Baird, LCDR, MSC, USN, NAMRU-2
Co-Investigators: Thomas L. Richie, LCDR, MC, USNR, NAMRU-2
Stephen L. Hoffman, CDR, MC, USNR

Objective: Determine the distribution and frequency of chloroquine-resistant *P. vivax* on the Indonesian archipelago, and examine alternative chemotherapeutic and chemoprophylactic regimens where resistance is highly prevalent.

Methods: An in vivo test for the parasitologic detection of resistance to chloroquine by *P. vivax* will be evaluated in northeastern Irian Jaya (known endemic resistance) and northwestern Sumatra (suspected endemic resistance). The test involves the administration of primaquine for causal prophylaxis during the 28 day post-chloroquine follow-up. The efficacy of primaquine for causal prophylaxis, and of halofantrine for therapy, against endemic chloroquine-resistant *P. vivax* will be evaluated using a placebo-controlled, double-blind fixed cohort analysis in Arso, Irian Jaya.

Progress: This laboratory has confirmed endemic resistance to chloroquine by *P. vivax* in Arso, Irian Jaya. In two separate studies, the 8 week cumulative incidence of *P. vivax* asexual parasitemia among people under supervised chloroquine prophylaxis (5 mg/kg/wk) was 45% (9 of 20 patients followed) and 67% (16 of 14 patients followed). In addition to at least 7 confirmed individual cases of *P. vivax* resistant to standard curative therapy with chloroquine, another study found the prevalence of resistance to be 22% (10 of 46 patients evaluated). In an experimental in vitro test of resistance to chloroquine by *P. vivax*, 5 of 11 strains evaluated developed schizonts in the presence of >4 pmole chloroquine per 50 ul well (12 to >64 pmole).

Plans: The studies to evaluate an in vivo test for chloroquine-resistant *P. vivax* employing primaquine prophylaxis will be completed during FY 92. The therapeutic trial of halofantrine, and the prophylactic trial of primaquine will be performed in early FY 93. Plans are now being developed for a systematic archipelago-wide survey of chloroquine resistance by *P. vivax* and *P. falciparum*. The survey consists of in vivo tests of resistance among 20 to 50 people at each of 6 sites within each of 4 separate zones representing all of Indonesia (480 to 1200 individual tests). The archipelago-wide surveys will begin in late FY 93 and be completed in early FY 95.

Publications:
1. Baird JK, Basri H, Purnomo, Bangs MJ, Subianto B, Patchen LC and Hoffman SL. Resistance to chloroquine by *Plasmodium vivax* in Irian Jaya, Indonesia. Amer J Trop Med Hyg 44:547-552, 1991.

2. Murphy GS, Basri H, Purnomo, Anderson EM, Bangs MJ, Mount DL, Ya-Ping S, Lal AA, Wiady I, Gorden J, Purwokusumo AR, Harjosuwarno S, Sorensen K and Hoffman SL. Vivax malaria resistant to treatment and prophylaxis with chloroquine. Manuscript submitted for publication.

Project Title: Development of a polymerase chain reaction (PCR) assay for *Plasmodium falciparum* malaria.

Funding: 63

Principal Investigator: Gary W. Long
Co-investigators: T.R. Jones,
L. Fries, Johns Hopkins University
S. L. Hoffman

Objective: To develop and evaluate a polymerase chain reaction assay for *P. falciparum* and devise methods to ease sample collection and processing.

Methods: We used the polymerase chain reaction to analyze blood specimens from subjects in a *P. falciparum* vaccine trial. The results were compared to the thick blood film

Progress: We developed a PCR assay with primers amplifying a 153 base pair region from the *P. falciparum* circumsporozoite protein gene. An oligonucleotide probe was labelled with digoxigenin-11-dUTP and used for chemiluminescent dot blot analysis of the results. Blood samples were collected on filter paper (5 μl) and stored dry at room temperature until analysis. PCR analysis was performed directly from the filter paper specimen. Ten subjects in the trial developed malaria, average parasitemia 16.8/μl (range 4-60) at time of blood film diagnosis. Eight of the ten were diagnosed by PCR from one to three days prior to blood film detection (average= 1.5 days); two were diagnosed on the same day as the blood film. There were eleven specimens positive by both blood film (average= 16 parasites/μl) and PCR. An additional 15 specimens were detected by PCR but were below the threshhold (<4 parasites/μl) for detection by blood film. The sensitivity of the assay exceeds that of the thick blood film and can be performed directly from blood spots collected on filter paper.

Plans: Further work is required to test the field applicability of the assay and to evaluate it in a controlled field trial.

Project Title: Evaluation of the Becton Dickinson QBC® system for the rapid diagnosis of malaria.

Funding: 63

Principal Investigator: Gary W. Long
Co-investigators: T.R. Jones,
L. Fries, Johns Hopkins University
J. Egan, WRAIR
S. L. Hoffman

Objective: To evaluate the sensitivity and specificity of the Becton Dickinson Quantitative Blood Count System (QBC®) for the rapid diagnosis of malaria.

Methods: We used the acridine orange/microhematocrit tube method (QBC®) for malaria diagnosis in ten *Plasmodium falciparum* vaccine and infectivity trials and compared it to the thick blood film.

Progress: Parasites were detected in 47 of the 49 subjects who became infected. Diagnosis was made by acridine orange from one to three days earlier than by blood film in 23 of the subjects (47%) and 20 were detected simultaneously with the thick blood film. Parasites were not observed in four subjects until twenty fours after diagnosis by blood film and diagnosis was not made in two subjects prior to blood film detection and treatment. Diagnosis was made by acridine orange at a parasitemia of less than or equal to 10 parasites per μl in more than two thirds of the cases. We conclude that the acridine orange assay is a rapid, accurate and easily performed alternative to the thick blood film for diagnosis of *P. falciparum* malaria.

Plans: Further work is required to: 1. determine the sensitivity and specificity of the test for other species of malaria; 2. evaluate the assay under field conditions; 3. evaluate the ability of medical corpsmen to use the test.

Publications:

1. Rickman L, Long G, Oberst R, Cabanban A, Sangalang R, Chulay, J, Hoffman S. Rapid diagnosis of malaria by acridine orange staining of centrifuged parasites. Lancet i: 68-71, 1989

2. Long G, Rickman L, Jones T, Trimmer R, Hoffman S. Acridine orange detection of *Plasmodium falciparum* malaria: relationship between sensitivity and optical configuration. Amer J Trop Med Hyg 44: 402, 1991.

3. Long G, Jones T, Rickman L, Fries L, Egan J, Wellde B, Hoffman S. Prepatant periods after experimental infection with *Plasmodium falciparum*. Submitted for publication.

4. Long G, Jones T, Rickman L, Fries L, Egan J, Wellde B, Hoffman S. Acridine orange diagnosis of *Plasmodium falciparum*: Evaluation in malaria vaccine trials. Submitted for publication.

Army Malaria Vaccine Program Review

FY92

Information Papers

Prepared by
Department of Immunology
Department of Bacterial Diseases
WRAIR

Information Paper

Department of Immunology
Walter Reed Army Institute of Research

18 May 1992

SUBJECT: Falciparum Malaria Vaccine

OBJECTIVE: Demonstrate the safety and efficacy of bio-engineered vaccine effective against 75% of falciparum malaria strains endemic in potential deployment areas of the world.

STRATEGY:

1. Identify candidate malaria antigens using animal and human models of preerythrocytic and blood stage immunity.

2. Construct bio-engineered vaccines that induce humoral and cellular responses correlated with protective immunity.

3. Conduct preclinical studies and clinical trials in humans to establish safety and efficacy.

CURRENT STATUS:

1. A panel of nine antigens have been identified as vaccine candidates from more than 30 cloned malaria antigens.

2. A series of bioengineered vaccines based upon the CS protein have been produced which induce humoral and/or cellular immunity directed against preerythrocytic stages.

3. Eleven Phase I studies have been completed, two are in progress, and three are scheduled for FY92 starts. Four vaccines proceeded to Phase IIa, and one to Phase IIb.

4. Clinically acceptable methods of inducing high levels of antibodies and CTL responses against liver stage parasites have been developed using liposomal encapsulation of malaria antigens.

5. Three bioengineered attenuated live vector systems for delivering multiple malaria antigens have been identified and are being used to construct vaccine for clinical studies.

TECHNOLOGICAL BARRIERS:

1. Expression of malaria genes in bioengineered systems is unpredictable.

2. Manufacturing capacity is a rate limiting step due to the low priority placed upon malaria vaccines by the industrial sector.

3. Animal models of falciparum malaria are limited and clinical trials using experimental and natural challenge of human volunteers are integral to the vaccine development process.

PLANS (to overcome barriers):

1. Multiple parallel approaches to expressing malaria genes are being developed including recombinant fermentation, peptide synthesis, and attenuated live vector systems.

2. In house capacity to produce pilot lot vaccines for IND studies is being enhanced through investment in facilities and equipment, and through leveraging of CRDA partners and non DOD Federal agencies involved in malaria vaccine development.

3. Enhanced capabilities to efficiently test malaria vaccines are being pursued within USAMRDC, through contractors, and in Special Foreign Activity field sites.

RESOURCES:

Additional personnel resources and funding to support their efforts are required to evaluate the large number of vaccine candidates currently being constructed. Existing personnel are insufficient if a truly effective malaria vaccine is to be available before FY98. Near term and long range programming of funds and personnel to support field studies must be urgently addressed.

W. RIPLEY BALLOU
LTC, MC
Program Manager
Army Malaria Vaccine Program
202-576-1257

INFORMATION PAPER

Department of Immunology
WRAIR

13 May 1992

SUBJECT: Plasmodium falciparum Vaccine Development

OBJECTIVE: Erythrocyte Binding Antigen-175 (EBA-175) a red cell invasion receptor : Characterization and identification of functional regions and development of EBA-175 as a malaria vaccine candidate.

STRATEGY:

1) Clone and sequence EBA-175 gene
2) Identify functional regions
3) Raise antibodies which block EBA-175 activity
4) Show protection in passive transfer experiments with these functional antibodies

CURRENT STATUS (See Appendix:1 for bibliography)

1) The gene encoding EBA-175 was cloned and characterized. EBA-175 possesses several predicted B and T cell (helper and cytotoxic) epitopes
2) Based on the deduced amino acid sequence, peptides predictive of antigenic determinants were synthesized. Antibodies were raised against these peptides in mice and rabbits.
3) The entire EBA-175 molecule was expressed in a bacterial system in overlapping pieces. Antibodies against these pieces were raised in mice.
4) The full-length EBA-175 gene was transfected into mammalian (monkey COS cells, mouse P815) cells to produce mammalian expressed full-length EBA-175 as well as to raise antibodies against the full length molecule.
5) An important objective is to identify regions on EBA-175 which are involved in binding to the red cell. Synthetic peptides, bacterial and mammalian cell expressed proteins representative of parts or the whole molecule are being analysed for their role in binding to erythrocytes.
6) A functional region (EBA-peptide 4, residues 1062-1103) encompassing 42 amino acids of EBA-175 has been identified.
7) Antibodies against EBA-peptide 4 :
   - block the binding of native EBA-175 to erythrocytes
   - inhibit the invasion of merozoites into erythrocytes
8) EBA-peptide 4 :
   - reverses the blocking of binding and reverses the inhibition of invasion
   - is conserved in all of 7 P.falciparum strains studied to date.
9) The fine specificity of antibodies against EBA-peptide 4 was examined. In an ELISA using a series of overlapping octapeptides spanning the entire EBA-peptide 4 as capture antigen, antibodies against EBA-peptide 4 were found to only react to a 19 amino acid region on EBA-peptide 4 (NEREDERTLTKEYEDIVLK, designated EBA-peptide 19)

10) EBA-peptide 19 :
- blocked the reactivity of EBA-peptide 4 antibodies to EBA-peptide 4 in a manner comparable to EBA-peptide 4 itself in competitive ELISA's
- EBA-peptide 19 also blocks IFA reactivity
- EBA-19 blocks the ability of EBA-peptide 4 antibodies to immunoprecipitate authentic EBA-175

11) Antibodies against KLS-19 :
- recognizes EBA-175 in immunoblots
- inhibits invasion of merozoites in vitro

12) The entire EBA-175 was expressed in overlapping fragments in a bacterial system. Antibodies eluted off immune clusters of merozoites which are directed against exposed epitopes on the merozoite recognised expressed fragment EBA-10.3 (amino acid residue 501-753) in immunoblots.

13) Antibodies raised against EBA-10.3 :
- recognizes merozoites in IFA
- recognizes EBA-175 in immunoblots

14) Synthetic peptides and expressed proteins are being used in live erythrocyte binding assays in both solid and liquid phases to determine other regions of EBA-175 which are involved in erythrocyte binding.

TECHNOLOGICAL BARRIERS:
1) Do not have monoclonal antibodies
2) Insufficient antibody production
3) Insufficient man-power on project

PLANS:
1) Contract out the task of monoclonal antibody production
2) Perform passive transfer experiments with purified Ig fraction of monoclonal antibody in Aotus monkeys
3) Study the fine specificity of EBA-175 binding to erythrocytes and look for other functional domains
4) Since EBA-175 is a liver stage antigen as well, the gene (or parts of eg. EBA-peptide 4) encoding EBA-175 will be cloned into vectors eg. Salmonella and studied for induction of cytotoxic T-cell activity in the mouse model initially [see number (5) of CURRENT STATUS]
5) The estimated time to achieve the objective of developing EBA-175 (or parts of) as a vaccine candidate is 1.5 years

RESOURCES/SPECIAL CONSIDERATIONS:
1) EBA-175 is the only malaria molecule that has been discovered, fully characterized and had functional regions identified all within WRAIR. It is truly a WRAIR molecule.
2) The project is not duplicated as yet by any other group elsewhere; the only reason being that WRAIR has had a head start in characterizing the gene encoding EBA-175
3) The project requires more man-power to achieve objectives sooner.

Kim Lee Sim Ph.D./(202)576-0867

Information Paper

Department of Immunology
Walter Reed Army Institute of Research

18 May 1992

SUBJECT: Molecular Mechanisms in Cerebral Malaria

OBJECTIVES:

1. Define the endothelial receptors responsible for sequestration at the molecular and cellular level.

2. Define the parasite-encoded counter-receptors on the infected erythrocyte surface which mediate binding to endothelial receptors.

3. Develop theraputic inhibitors of malaria sequestration for cerebral and severe malaria.

4. Develop a vaccine candidate antigen which prevents parasite sequestration.

STRATEGY:

1. Endothelial Cell Receptors
   a. CD36
   b. ICAM-1
   c. ELAM-1
   d. VCAM-1

2. Parasite Counter Receptors
   a. Malaria parasite protein "sequestrin"
   b. Other parasite proteins ?

3. Theraputic Interventions
   a. Soluble peptides from ICAM-1
   b. Immunoadhesins

4. Vaccine Development
   a. Use of anti-sequestrin antibodies or Immunoadhesins to identify relevant parasite molecules for inclusion in a vaccine.

   b. Identify relevant parasite DNA sequences to molecularly clone and express vaccine candidate antigens.

CURRENT STATUS:

The human endothelial cell antigens CD36, ICAM-1, ELAM-1 and VCAM-1 have been identified as receptors for attachment of Plasmodium falciparum infected erythrocytes. Using an anti-idiotypic antisera prepared against a monoclonal antibody against CD36 (the anti-idiotypic antibody mimics CD36), we identified a >200 kD parasite protein (called sequestrin) as a counter receptor of CD36. The binding site on ICAM-1 has been localized to its first domain and soluble peptides from ICAM-1 inhibit binding of infected erythrocytes. The binding domain of ICAM-1 was

recombinantly expressed in tandem with the constant domains of human IgG1. This chimeric "Immunoadhesin" coats infected erythrocytes and promotes phagocytosis by human macrophages.

TECHNOLOGICAL BARRIERS:

1. Insufficient quantities of endothelial receptors
2. Poor immunogenicity of sequestrin
3. Potential reactogenicity of soluble peptides

PLANS:

1. Recombinantly express endothelial receptors in eucaryotic expression systems.

2. Identify sequestrin by screening DNA libraries with anti-idiotype.

3. Use immunoadhesins to reverse sequestration.

4. Evaluate immunoadhesins in primate models for malaria.

RESOURCES:

1. Institutional commitment

2. Large scale eucaryotic gene expression systems suitable for GMP level of production.

CPT(P) Francis W. Klotz/576-3780

Information Paper

Department of Bacterial Diseases
Walter Reed Army Institute of Research

18 May 1992

SUBJECT: Live-attenuated *Salmonella* Vaccines Expressing Malaria Antigens.

OBJECTIVE: Develop live-attenuated strains of *Salmonella typhi* that stimulate protective immunity in humans.

STRATEGY:

1. Construct attenuated strains of *S. typhi* that are well tolerated and stimulate protective immunity in humans.

2. Express Malaria antigens in *S. typhi*.

3. Evaluate immune response to vaccine in animals and humans.

CURRENT STATUS:

1. *S. typhi* 101018 was attenuated by introducing *AroA* mutation.

2. Circumsporozoite surface protein (CSP) , Erythrocyte binding protein (EBA), and a unique EBA/CSP fusion protein have been cloned and expressed with plasmid cloning vectors in *S. typhi*.

3. EBA peptide 4 was integrated into *srbC* chromosomal locus.

TECHNOLOGICAL BARRIERS:

1. Plasmid vectors are unstable in *S. typhi*.

2. *AroA* may not attenuate *S. typhi* sufficiently for human use.

3. Malaria antigens may not be processed or properly presented to immune system.

PLANS (to overcome barriers):

1. Integrate malaria genes in the chromosome.

2. Add additional attenuating mutations to vaccine strains (*galE*, *AroC/D*, *cya/crp*, or *recA*).

3. Express malaria genes on the surface of *S. typhi*, construct novel fusion peptides that carry B and T-cell epitopes.

RESOURCES:

A core of research molecular biologist has recently been recruited and are devoting 100% of their efforts to developing a live-attenuated *S. typhi* vaccine strain for the stimulation of protective immunity to malaria in humans. Strain construction and the evaluation of the immune response to these vaccine constructs is labor intensive and requires well trained professionals.

Richard L. Warren, Ph.D./202-576-3147

INFORMATION PAPER

Department of Immunology
Walter Reed Army Institute of Research

18 May 1992

SUBJECT: Expression of Plasmodium falciparum genes in vaccinia vector.

OBJECTIVE: To evaluate the use of vaccinia virus as a vector for the expression of malaria genes in a human use vaccine.

STRATEGY: (work done in collaboration with Virogenetics Corporation, Troy, NY. under a CRADA)

1. Create an attenuated vaccinia virus that would not replicate in human cells but would express extrinsic antigens. (work done by Virogenetics)

2. Clone seven malaria genes into this vector both individually and all together as a multicomponent vaccine. (Work done at WRAIR and Virogenetics). Each gene would be under control of separate poxvirus promoters. The seven genes selected would be representative of different stages of parasite development. They would include:

    1. CS (Sporozoite)
    2. SSP2 (Sporozoite)
    3. LSA (Liver)
    4. MSP 1 (Merozoite)
    5. SERA (Merozoite)
    6. AMA 1 (Merozoite)
    7. PF-25 (Ookinete)

3. Evaluate expression of each gene in cultured cells and in rabbits and mice. (Work done at Virogenetics and WRAIR)

4. Evaluate use of this recombinant vector in human use studies. (Work done at WRAIR)

CURRENT STATUS:

1. A highly attenuated vaccinia virus vector, NYVAC, has been made from the Copenhagen vaccine strain by the precise deletion of 18 open reading frames from the viral genome. The NYVAC virus has no detectable induration or ulceration at the site of inoculation, rapid clearance of infectious virus, greatly reduced virulence, and greatly reduced pathogenicity yet still induces strong immune responses to extrinsic antigens.

2. Five of seven genes have been cloned into individual NYVAC virus and into the multicomponent NYVAC 7 virus.

The two remaining genes, SSP2 and LSA, have been cloned into shuttle vectors and are presently being introduced into the vaccinia by in vivo recombination. All genes that have been cloned into individual vaccinia viruses show gene expression in cultured cells and in rabbits and mice. The multicomponent NYVAC has not been tested yet.

TECHNOLOGICAL BARRIERS:

1. Limited immunological reagents for identification of cell mediated immune responses to expressed antigens in animal models.

2. Gene variation among malaria in the natural population and the ability of malaria parasites to undergo antigenic variation both naturally and under immune pressure.

PLANS:

1. To develop permanently transformed cells lines expressing each of the seven malaria genes selected in our study. This will be done with the use of retroviral expression vectors under a CRADA with Viagene of San Diego, CA. (see additional Information Paper). These cells will function as antigen presenting cells and target cells in various cell mediated immune response studies.

2. The problem of gene variation hopefully will be overcome using the multicomponent NYVAC 7 vector. The genes that have been chosen for inclusion into this study are ones that express limited antigenic variation in nature. By stimulating the immune response to a variety of antigens at once we hope to increase the chances of correctly choosing genes expressed in the wild and limiting the ability of the parasite to evade the immune response to all the genes by antigenic variation before it is eliminated from the host.

3. NYVAC vectors containing malarial genes will be constructed in 2 months; complete in vivo and in vitro evaluation will be completed in 6 months; phase I human studies will be completed in 18 months.

RESOURCES NEEDED:

1. Capillary electrophoresis machine to quickly evaluate oligonucleotide synthesis and purified proteins.

David Lanar, GS-14
Gray Heppner, MAJ,MC
202-576-3331

INFORMATION PAPER

Department of Immunology
Walter Reed Army Institute of Research 18 MAY 1992

SUBJECT: Expression of Plasmodium falciparum genes in BCG vector.

OBJECTIVE: To evaluate the use of BCG as a vector for the expression of malarial genes in a human use vaccine.

STRATEGY: (work done in collaboration with MedImmune Corporation of Gaithersberg, MD. under a CRADA)

1. Create a series of plasmid vectors that grow and express genes in BCG. (work done by MedImmune Corp.)

2. Clone a variety of malarial genes into these vectors and evaluated for expression levels. (Work done at WRAIR) Malarial genes will include:
    1. CS - full length
    2. CS - repeatless
    3. CS - 6R C-term (6 repeats and C terminal region)
    4. SERA
    5. SSP2
    6. MSP 1
    7. EBA-175
    8. LSA
    9. AMA 1

3. Evaluate the ability of the vectors expressing malarial genes to induce immune responses in animal models. (Work done at WRAIR and MedImmune)

4. Evaluate the use of these recombinants in human use studies (work done at WRAIR)

CURRENT STATUS:

1. Ten plasmid vectors have been developed by MedImmune Corporation for the cloning of genes. These vectors differ from each other by their promoters and signal or leader sequences. Some make fusion proteins and some make native proteins. These plasmids grow in E. coli and BCG.

2. In each of these ten plasmids we have cloned the following genes:
    1. CS - full length
    2. CS - repeatless
    3. CS - 6R C-term (6 repeats and C terminal region)
    4. SERA
    5. SSP2
    6. MSP 1

    The expression levels of these genes is currently being evaluated. CS - full length does not express in any

construct; CS - repeatless and CS 6R C-term each express in 4 plasmids; SERA expresses in one plasmid. SSP2 and MSP 1 are still being grown in BCG.

3. BCG-malarial constructs that do express malarial genes, as detected by Western Blot, are being grown for injection into animal models.

TECHNOLOGICAL BARRIERS:

1. Limited immunological reagents for identification of cell mediated immune responses to expressed antigens in animal models.

2. Inability to predict which plasmid promoters and leader sequences will best be suited for expression in BCG.

3. Slow doubling time of BCG (>24 hrs) means long times for growing cells. Therefore many combinations are tried at once without waiting for results. Involves a large amount of manpower.

PLANS:

1. To clone each of the selected malarial genes into at least one BCG compatible plasmid.

2. To develop permanently transformed cell lines expressing each of the genes selected in our studies. This will be done with the use of retroviral expression vectors under a CRADA with Viagene Corporation of San Diego, CA. (see Information Paper.) These cells will function as antigen presenting cells and target cells in various cell mediated immune response studies.

3. Cloning and expression level evaluation will be completed in 18 months.

RESOURCES NEEDED:

1. Animals, both mice and rabbits, to evaluate in vivo expression levels. Additionally, animal facilities at WRAIR are inadequate to handle the large number of mice and rabbits needed in these studies. Outside contractors, which are available, need to be used but no money exists to hire them.

David Lanar, GS-14
Gray Heppner, MAJ,MC
202-576-3331

INFORMATION PAPER

Department of Immunology
Walter Reed Army Institute of Research 18 MAY 1992

SUBJECT: Expression of Plasmodium falciparum genes in retroviral vector.

OBJECTIVE: To clone malarial genes or parts of genes into retroviral vectors for establishing permanently transformed expressing cell lines.

STRATEGY: (work done in collaboration with Viagene Corporation of San Diego, CA under a CRADA)

1. Genetically engineer retroviral shuttle plasmid KT-3 to accept malarial genes. (Plasmid KT-3 supplied to WRAIR; genetic engineering done at WRAIR)

2. Clone a variety of malarial genes into this vector and evaluated for expression levels. (Work done at WRAIR) Malarial genes will include:
    1. CS - full length
    2. CS - repeatless
    3. CS - 6R C-term (6 repeats and C terminal region)
    4. SERA
    5. SSP2
    6. MSP 1 (fragments)
    7. EBA-175
    8. LSA
    9. AMA 1

3. Evaluate the ability of this vector to express malarial genes. (Work done at WRAIR and Viagene)

4. Evaluate the use of these recombinants in human use studies as Antigen Presenting Cells and Target cells in various cell immune response studies on a variety of malarial antigens. (work done at WRAIR)

CURRENT STATUS:

1. The KT-3 shuttle plasmid has just been received at WRAIR and genetic re-engineering is proceeding.

TECHNOLOGICAL BARRIERS:

1. Inability to predict whether plasmid promoters will express malarial genes incorrectly or whether malarial proteins will be toxic to host cells.

PLANS:

1. To clone each of the selected malarial genes into the

adapted KT-3 plasmid.

2. To develop permanently transformed cell lines expressing each of the genes selected in our studies. This will be done at Viagene in San Diego, CA.

3. Cloning and expression level evaluation will be completed in 18 months.

RESOURCES NEEDED:

1. Greatly expended tissue culture facility will be needed as well as increased funds for materials needed for continual propagation of transfected cell lines.

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202-576-3331

Information Paper

Dept. of Immunology
WRAIR

17 May 1992

SUBJECT: *P. falciparum* Vaccine Development

OBJECTIVE: To evaluate cell mediated immunity induced with (a) recombinant subunit vaccines or (b) live vector vaccines.

STRATEGY:

1. Testing of circumsporozoite (CS) protein subunit based vaccines

   A. *P. falciparum* repeatless (RLF) SmithKline Pharmaceuticals
      a). Liposomes as adjuvants -delivery system
      b). Induction of cytolytic T lymphocytes (CTL)*in vivo* (rodent model)
      c). Target specificity of CTL's

   B. RLF vaccine trail in human volunteers
      a). Evaluation of CTL
      b). Evaluation proliferative T cells
      c). Evaluation of T cell subsets by lymphokine production

   C. RTS,S (recombinant CS protein-hepatitis particles construct)SmithKline Pharmaceuticals
      a). Application of different adjuvants, i.e. alum, MPL
      b). Induction of cytolytic T lymphocytes (CTL)*in vivo* (rodent model)
      c). Target specificity of CTL's

   D. RTS,S vaccine trial in human volunteers
      a). Evaluation of CTL
      b). Evaluation proliferative T cells
      c). Evaluation of T cell subsets by lymphokine production

2. Evaluation of vaccinia (Virogenetics) vectors containing malaria target antigens in the induction of CTL responses in volunteers immunized with SPZ and subunit vaccines.

   A. Vaccinia-CS protein construct
      a). application as target antigens in the CTL assay
      b). application as stimulating antigens *in vitro* CTL induction

   B. Vaccinia-MSP1 construct
      a). application as target antigens in the CTL assay
      b). application as stimulating antigens *in vitro* CTL induction

   C. Vaccinia-LSA construct
      a). application as target antigens in the CTL assay
      b). application as stimulating antigens *in vitro* CTL induction

   D. Vaccinia-SERA construct
      a). application as target antigens in the CTL assay
      b). application as stimulating antigens *in vitro* CTL induction

   E. Multitarget vaccine- NYVAC construct
      a). application as target antigens in the CTL assay

b). application as stimulating antigens *in vitro* CTL induction

3. Evaluation of retroviral (Viagene) vectors containing malaria target antigens in the induction of CTL responses in volunteers immunized with SPZ and subunit vaccines. (as in #2)

4. Evaluation of BCG (MedImmune) vectors containing malaria target antigens in the induction of CTL responses in volunteers immunized with SPZ and subunit vaccines. (as in #2)

CURRENT STATUS:

1. CS protein subunit based vaccines: Immunization with liposome-encapsulated RLF, but not with RLF alone induces murine CTL responses that are directed to the *P. falciparum* CS protein epitope a.a. residue 368-390 and also recognize CS protein gene transfected MHC compatible cells. The CTL are $CD8^+$ and lyse the target cells in MHC class I restricted fashion.

Preparations for RLF vaccine trials in human volunteers are in progress. Assays and reagents for the evolution of celluiar responses are available.

RTS,S vaccine induces CTL responses in mice. Currently, the adjuvants systems and the specificities of CTL are being evaluated. Concurrently, CTL responses in human volunteers immunized with the RTS,S vaccines are being evaluated. Analysis of proliferative responses to selected synthetic peptides of CS protein and lymphokine production are in progress.

2. Vaccinia-malaria target antigen constructs: Vaccinia vectors expressing *P. falciparum* CS protein have been now routinely used as targets in CTL assays testing peripheral blood lymphocytes from volunteers participating in vaccine trials, including irradiated SPZ and RTS,S. These constructs have also been used as stimulators in the *in vitro* induction of CTL.

Currently, preparations are being made to utilize the vaccinia-MSP1 and vaccinia-LSA constructs to test for the induction of exoerythrocytic stage antigen-specific CTL's in the irradiated SPZ volunteers.

3. Retroviral-malaria target antigens: Experiments are in the planning phase.

4. BCG-malaria target antigens: Constructs containing fragments of the CS protein are available and plans are being made for testing of the constructs in the induction of CTL responses in mice.

TECHNOLOGICAL BARRIERS:

1. Insufficient personnel trained to perform these complicated cellular assays
2. Insufficient funds for testing of the vaccines in the rodent model
3. Insufficient laboratory space and equipment to handle the volume of the work

PLANS:

To increase the personnel capable of handling these experiments.

RESOURCES:

A successful accomplishment of this objective will require an additional personnel consisting of postdoctoral fellows with a background in cellular immunology.

Urszula Krzych, Ph.D.
Dept. Immunology
WRAIR

Information Paper

Dept. of Immunology
WRAIR

17 May 1992

SUBJECT: *P. falciparum* Vaccine Development

OBJECTIVE:

To evaluate the role of cellular responses mediating protective immunity induced with radiation-attenuated sporozoites (SPZ) in the murine model of *P. berghei.*

STRATEGY:

1. Identification of the T cell populations responding to SPZ antigens
   - A. Proliferative T lymphocytes
   - B. Cytolytic T lymphocytes
   - C. T helper (Th)1 and 2 subsets by lymphokine analysis
     - a. Th1- lymphokine analysis for IL-2, IL-3, and IFN-γ
     - b. Th2-lymphokine analysis for IL-4 and IL-6

2. Processing and presentation of SPZ antigen
   - A. Identification of CS protein-reactive T cells
     - a. SPZ immunization
     - b. CS protein immunization
   - B. Presentation of SPZ antigen
     - a. Antigen presenting cells
     - b. Mechanism of presentation
   - C. The role of cryptic epitopes on CS protein
     - a. Immunization with synthetic peptides
     - b. presentation of cryptic epitope peptides
     - c. Functional specificity of cryptic epitopes

CURRENT STATUS:

1. SPZ reactive T cells: A) SPZ immunization induces specific T cells reactive to the native SPZ antigen, recombinant full length CS protein,but not to synthetic peptides of CS protein. B). CTL's reactive to Pb CS protein peptide a.a. residue 249-261 and to *P. berghei* CS protein gene transfected fibroblasts are induced in Balb/c mice following SPZ immunization. C). Lymphokine analysis reveals that both Th1 and Th2 cells are induced following SPZ immunization. There are differences in the time of induction among the murine strains indicating the strain and SPZ-dose dependent protection may result form differential T cell induction. Th2 cell induction accompanied protection and the precise role of these cells in protection will be investigated.

2A. Processing and presentation of SPZ antigens. SPZ-immune lymphocytes proliferate to the priming antigen and to recombinant, soluble CS (rCS) protein, CS protein synthetic peptides do not recall reactivity. Parallel analysis of rCS protein-primed lymphocytes revealed that the two antigens are unequal in generating T cell specificities: while SPZ priming did not induce CS protein specific peptide-reactive T cells, priming with rCS protein did. The hypothesis that each form of the SPZ antigen is subject to a unique antigen processing was also confirmed in experiments demonstrating a lack of recognition of the authentic CS protein by rCS protein-primed lymphocytes.

2B. To discern distinct T cell specificities induced with SPZ-associated vs. peptide or rCS protein, the presentation of SPZ-associated CS protein was investigated, indicating that B cells are efficient accessory cells in this response and macrophages are not involved in anti-SPZ proliferative reactivity. Analysis of the presentation mechanism by processing inhibitors (gluteraldehyde, monensin) revealed that SPZ-specific T cell activation by SPZ-associated CS protein is not dependent upon intracellular processing by the presenting B cell.

2C. Since an extensive CS protein T cell repertoire is available, the role of T cells directed against the cryptic epitopes of the CS protein was investigated. Cryptic T cell epitopes were analyzed utilizing overlapping 20-mer peptides of the CS protein, and the immunogenic regions mapped to the repetitive sequence as well as regions of homology within the Plasmodium genus. A T cell clone specific for the repeat peptide recognized not only the priming peptide, but also the native SPZ antigen. Both antigens were recognized in a class II restricted manner, and cytolytic activity against B cells pulsed with SPZ or the peptide was observed. Lymphokine analysis demonstrated that the peptide induces the clone to produce IL-4, while SPZ do not. These observations suggest that the CS protein peptides prime T cells capable of recognizing SPZ antigens, however, these cells are not expanded sufficiently upon SPZ immunization, perhaps due to an immunodominant conformational determinant presented by activated B cells.

TECHNOLOGICAL BARRIERS:

1. Limited personnel
2. Limited funds to purchase supplies
3. Difficulties inherent to the assay system
4. Limited equipment

PLANS:

1. Alternative means of support

RESOURCES:

Currently this project is being accomplished by two doctoral students supported by a WHO/World Bank grant. One student is expected to graduate within the next 6 months. The continuation of this project will necessitate another person with experience in cellular immunology and molecular biology, preferably a postdoctoral fellow. In addition, equipment including a fluorescent activated cell sorter (FACS) is necessary. Finally, sufficient funds to purchase supplies such as tissue culture reagents are essential.

Urszula Krzych, Ph.D./(202)576-3364

Information Paper

Dept. of Immunology
WRAIR

17 May 1992

SUBJECT: *P. falciparum* Vaccine Development

OBJECTIVE: To evaluate the role of cellular responses mediating protective immunity induced with radiation-attenuated sporozoites.

STRATEGY:

1. Identification of the targets of sporozoite-induced T cell

   A. Circumsporozoite (CS) protein antigens
      a. Full length recombinant CS protein (Chiron)
      b. Repeatless CS protein (RLF) (SKB)
      c. CS protein peptides (WRAIR)

   B. Exoerythrocytic antigens
      a. MSP 1 fragments
      b. MSP 2
      c. MSP 2 peptides
      d. LSA 1
      e. SERA

   C. pRBC lysates

2. Analysis of sporozoite-induced T cell subsets

   A. Monitoring of $CD4^+$ and $CD8^+$ T cells subsets
   B. Induction of memory markers on T cells
   C. T cell receptor gene families usage

3. Analysis of cellular effector mechanism(s)

   A. Cytotoxic T cells (CTL)
      a. specific for CS protein peptide a.a. residue 368-390
      b. specific for CS protein peptide a.a. residue 331-350

   B. Evaluation of live vector constructs as targets for CTLs
      a. Vaccinia-CS protein
      b. Vaccinia-MSP 1
      c. Vaccinia-LSA 1
      d. Vaccinia-SERA
      e. Vaccinia (NYVAC)-multiple constructs

4. T cell cloning and analysis of their antigen specificities and functional reactivities.

CURRENT STATUS:

1. Targets of sporozoite induced T cells: (1) Sporozoite immunization induces CS protein-reactive T cells in some volunteers, but the proliferative responses do not correlate with protection. (2) Anti-CS protein peptide reactive T cells have not been observed at any time after sporozoite immunization or challenge. (3) In contrast, proliferative responses to the exoerythrocytic antigens as well to antigens found on the lysates of parasitized erythrocytes have been observed in sporozoite immune/protected volunteers. Although minor responses were noted in the preimmune lymphocyte populations, they increased with each immunization. Responses to parasitized erythrocytes were not observed in lymphocytes obtained from a single nonprotected volunteer and from naive control lymphocytes.

2. Analysis of T cell subsets: In its initial phase of the study,this project is being conducted in collaboration with colleagues at NIH.

3. Effector Mechanisms: CTL responses specific for the PfCS protein peptide 368-390 have been measured using preimmune and sporozoite-immune lymphocytes obtained from selected donors. The results demonstrate that circulating CTLs appear in the peripheral blood lymphocytes (PBL) of volunteers between days 5 - 12 after sporozoite immunization. The appearance of CTL's parallels the development of liver stage parasites following sporozoite inoculation and the expression of liver stage antigens, including CS protein. To date we have successfully analyzed vaccinia vectors expressing CS protein as targets for CTL's. The remaining vectors are being analyzed. CTL with specificities other than PfCS protein peptide 368-390 have not been found.

4. T cells specific for fragments of MSP 1 have been cloned and their functional specificities will be tested.

TECHNOLOGICAL BARRIERS:

1. Limited personnel
2. Limited number of lymphocytes/volunteers
3. Limited funds for basic research
4. Limited equipment (FACS)

PLANS

1. Initiate collaborative projects with other laboratories
2. Recruitment of a larger number of volunteers
3. Alternative means of support (grants)

RESOURCES

Successful accomplishment of this objective will require an additional personnel consisting of postdoctoral fellows with a background in cellular immunology. Furthermore, to complete the phase on the identification of T cell subsets, a fluorescence activated cell sorter (FACS) or at least a cytofluorometer is essential.

Urszula Krzych, Ph.D.
Dept. Immunology
WRAIR

Information Paper

Department of Immunology
Walter Reed Army Institute of Research

18 May 1992

SUBJECT:
Clinical testing of malaria vaccine candidates (Phase I thru Phase IIb)

OBJECTIVE:
To systematically evaluate safety, immunogenicity and preliminary efficacy of potential malaria vaccine candidates in human volunteers.

STRATEGY:
1. Based on pre-clinical safety, immunogenicity, and (when applicable)*in vitro* growth inhibition / inhibition of invasion assays, select vaccine candidates, prepare. assemble, and submit application for Investigational New Drug Exemption.

2. Conduct Phase I safety and immunogenicity studies either through :
Clinical Center, WRAIR
Medical Unit, USAMRIID
associated NMRI Facilities
other local military installations
local university based vaccine test centers via contract

3. Conduct Phase IIa safety, immunogenicity, and efficacy studies using laboratory challenge model. Source of infectious mosquitoes (*P. falciparum* NF54, 3D7, or 7G8):
Department of Entomology, WRAIR
NMRI associated insectary

Potential future test sites:
Vaccine Trial Center
Bangkok Hospital for Tropical Diseases
Mahidol University
Bangkok, Thailand

4. Conduct Phase IIb safety, immunogenicity, and efficacy studies under conditions of natural exposure. Current available test sites include:
Military components, Royal Thai Army
Shoklo Camp, Mae Sod, Thailand thru Bangkok Hospital for Tropical Diseases, Mahidol University
Sarradidi Rural Health Facility thru USAMRU-K and the Kenya Medical Research Institute.

CURRENT STATUS:
Phase I studies:
NS1(81) RLF, (*P. falciparum* CS repeatless) pending IND submission, anticipated start date = July 92

SPf66, (Multistage *P. falciparum* vaccine) pending IND submission, anticipated start date = July 92

SPf66, AFRIMS/Mahidol Univeristy, Phase I protocol in preparation, in anticipation for Phase IIa/Phase IIb trials in Thailand

Pfs25 (*P. falciparum* Transmission Blocking Vaccine)
Phase I protocol in preparation

Phase IIa studies:
Liposome encapsulated R32NS1 Pending addendum to FDA
anticipated start date = July 92

Phase IIb studies:
R32ToxA in Thailand & Kenya - studies complete data analysis in progress

TECHNOLOGICAL BARRIERS:
- Source for GMP quality material for Phase I and IIa studies
- *In vitro* assays predictive of protection

PLANS:
- In house GMP peptide/recombinant protein production & purification plant; live vector production facility
- Improve in house animal resources to handle rabbit requirements for pre-clinical safety/immunogenicity data and product potency/stability
- Clinical Center, WRAIR expansion and training of 8N & 8Z personnel
- Initiate additional epidemiological evaluations of potential *Phase IIb test sites*

RESOURCES:
- Need facilities capable of conducting Phase I & IIa studies, limitations linked both to facility plant (insectary), and personnel with entomological and clinical expertise as well as volunteer availability for malaria challenges
- Need for additional field test sites with well doccumented transmission patterns
- Anticipated funding requirements for:

Product / Phase / Site:	Funding for:
NS_{81}RLF / Phase I / USAMRIID	FY92
R32NS1 (Liposome) / Phase I, IIb /	FY93
SPf66 (Alum) / Phase I / WRAIR	FY92/F93
SPf66 (Alum) / Phase I, IIb / AFRIMS	FY92/FY93
SPf66 (Liposome) / Phase I / WRAIR	FY93
RTS,s (Alum/3DMPL) / Phase I, IIb / AFRIMS	FY92/FY93
RTS,s (Liposomes) / Phase I, IIa / USAMRIID	FY93
RTS,s (Liposomes) / Phase I, IIb / AFRIMS	FY93
Pfs25 (Alum) / Phase I / WRAIR & NIH	FY92/FY93
PFs25 (Alum) / /Phase I, IIb / USAMRU-K	FY93

LTC Daniel M. Gordon/DSN 291-3419

Information Paper

Department of Immunology
Walter Reed Army Institute of Research

14 May 1992

SUBJECT: Cloning and Expression of P. falciparum Candidate Vaccine Antigens in Bacterial Hosts

OBJECTIVE: To evaluate various Escherichia coli expression vectors and hosts to obtain maximum production of P. falciparum antigens; purification of expression products.

STRATEGY:

1. Evaluate various E. coli expression vectors
   a. pTRC99
   b. pRX
   c. pT7-7
   d. pGEMX1

2. Evaluate host strains
   a. HB101
   b. DH5
   c. K38
   d. SC122 vs K165

3. Growth conditions
   a. Vary growth temperature
   b. Adding Rifampin to T7 expression clones after induction
   c. Vary growth media and composition

4. Develop vector for use of recombinant products in humans

CURRENT STATUS:

1. Vector - use pT7 and pGEMX1 vectors; high yield and specifically label expression product.

2. Host - use K165 due to decreased degredation of protein (see attached reprint, Lindler, Anders and Herman, 1991, Protein Expression and Purification, 2:321).

3. Growth Conditions - lowered growth temperature decreases protein degradation; Rifampin reduces protein background.

4. Vector for Human Use - replaced ampicillin resistance gene with chloramphenicol resistance in pT7-7 vector.

TECHNOLOGICAL BARRIERS:

1. Inadequate vetcor for expression of non-fusion malarial proteins.
2. Time consuming DNA sequencing of cloned products.
3. Lack of immunological reagents for identification of clones.

PLANS:

1. Develop T7 promotor based vector using SmithKline vector based protein fusions.

2. DNA sequencing gel reader.

3. Use radiolabeled expression product to aide in purifying antigen.

4. Time table.

   a. develop vector - 6 months
   b. gel reader - 1 year
   c. purification - 1 year

RESOURCES:

1. DNA sequencing gel reader.
2. Technician for fermentation and purification.
3. Columns and accessories for HPLC.
4. Water purification system.

Luther Lindler, Ph.D./DSN

Information Paper

Department of Bacterial Diseases
Walter Reed Army Institute of Research

18 May 1992

SUBJECT: Peptide Production under Good Manufacturing Practices (GMP) Conditions

OBJECTIVE: To establish a laboratory in which peptides can be reproducibly manufactured under GMP conditions.

STRATEGY:

1. Remodel laboratory in Building 508 for GMP manufacture.
2. Acquire laboratory equipment for laboratory.
3. Set up Standard Operating Procedures (SOPS) for each procedure.
4. Manufacture GMP quality peptides.

CURRENT STATUS:

1. *Building 508 plans completed.* Contractor has set potential delivery date for end of May 1992.
2. Majority of equipment purchased and stored in Bldg 505.
3. Initial GMP training started.

TECHNOLOGICAL BARRIERS:

1. Scale-up of research production.
2. Analytical quality control procedures.
3. Inadequate skilled personnel.

PLANS (to overcome barriers):

1. Purchase of large scale reaction vessels and testing of large scale cycle production.
2. Establish gas chromatography procedures to analyze organic chemicals.
3. Identify an alternative method for staffing the facility such as a contractor or CRDA personnel.

RESOURCES:

The special requirement in GMP production is for adequate skilled personnel to fully document all phases of production and to perform the analytical and product quality analyses. This is an extremely labor intensive area. The analytical procedures are more extensive than for laboratory work and therefore will require additional funding compared to the same level of research production.

Carolyn D. Deal, Ph.D./202-576-3601

Information Paper

Department of Immunology
Walter Reed Army Institute of Research

18 May 1992

SUBJECT: P. falciparum Vaccine Development

OBJECTIVE: To develop a monkey model for the preclinical testing of blood stage vaccine candidates against P. falciparum.

STRATEGY:

1. Establish baseline data for P. falciparum infections in Aotus nancymai.
2. Actively immunize monkeys with candidate vaccines using single synthetic peptides, combinations of peptides or recombinant polypeptides. Determine safety and immunogenicity of vaccines and response to challenge with P. falciparum.
3. Passively immunize monkeys with homologous immune globulin. Develop test systems for heterologous globulin (mice, rabbit & human) prepared by artificial immunization or affinity purification.
4. Attempt to correlate results from monkeys with in vitro methods such as "growth inhibition" and "cytoadherence" assays.

CURRENT STATUS:

1. A dependable model for immunity to P. falciparum has been established using Aotus nancymai. The monkey is sensitive to the protective blood stage antigens of P. falciparum and responds to infection with a long lasting immunity which is primarily antibody mediated.
2. Monkeys have been imunized with a combination of four synthetic peptides of the RESA antigen coupled to proteosomes; two different recombinant polypeptides of the Gp195 surface protein and peptide-4 of the Erythrocyte Binding Antigen. All vaccines induced moderate to high antibody titers when administered with Freund's adjuvant, however all failed to significantly protect Aotus nancymai against challenge with P. falciparum.

3. The gamma globulin of monkeys recovered from infection confers a marked resistance to naive monkeys when passively transferred prophylactically or against established infections. Homologous antibody also reverses cytoadherence. RESA peptide-affinity purified human hyperimmune globulin failed to moderate infections in monkeys.

TECHNOLOGICAL BARRIERS:

1. Intermittent supply of monkeys and inadequate primate facilities. Lack of continuity in monkey care and maintenance.
2. Use of feral animals include problems of other diseases which may influence malaria infections.

PLANS (to overcome barriers):

1. Screen newly arrived monkeys for important diseases thought to interfere with malaria.

RESOURCES:

1. Continued supply of Aotus monkeys designated for the Department of Immunology.
2. Assignment of a Veterinary Technician to the Department of Immunolgy.

Bruce T. Wellde, Ph.D./576-4206

Information Paper

Department of Immunology
Walter Reed Army Institute of Research

18 May 1992

SUBJECT: P. falciparum Vaccine Development

OBJECTIVE: To develop in vitro assays believed to correlate with antibody-mediated immunity to P. falciparum in Aotus monkeys, and to use these assays to screen mouse hybridomas for protective antibodies.

STRATEGY:

1. Reversal of Cytoadherance: to determine conditions for a simple, reproducible assay of cytoadherence reversal.

2. *Inhibition of Parasite Growth In Vitro:* to determine conditions for a simple, reproducible assay of inhibition of parasite growth in vitro.

3. Control Sera: to develop positive and negative control sera from Aotus monkeys to be used in these assays.

CURRENT STATUS:

1. Reversal of Cytoadherence: numerous variables (time & temp. of incubation, washing procedures, fixitives, etc.) are being evaluated for three different target cell lines (the C32 and EA-hy 926 transformed melanoma and endothelial cell lines, and non-transformed umbilical vein endothelial cells).

2. Growth Inhibition In Vitro: the evaluation of numerous variables (volume of culture, % hematocrit, % parasitemia, value of daily media change, etc.) for growth of P. falciparum in microtiter plates is nearly complete.

3. Positive (immune) control sera have been developed against the CAMP strain of P. falciparum and shown to be protective in passive transfer experiments in Aotus monkeys.

TECHNOLOGICAL BARRIERS:

1. Reversal of Cytoadherence: a method for screening hundreds of samples quickly and easily must be developed for screening hybridoma supernatants. This requires a method whereby the target cells can be fixed, (dried?), stored, and used at a later date.

PLANS (to overcome barriers):

1. Reversal of Cytoadherence: a variety of fixatives are being evaluated in conjunction with a variety of cultureware to develop a method for fixing target cells for later use.

RESOURCES:

1. Large quantities of tissue culture reagents, media, sera and plasticware are required to complete this project.

2. Human AB serum and special endothelial growth factors are required to grow and maintain the non-transformed umbilical vein endothelial cell line.

Ted Hall, Ph.D./576-3760

Information Paper

Department of Immunology, Div. CD&I
Walter Reed Army Institute of Research

19 May 1992

SUBJECT: Parasite production and invasion assays in support of *P. falciparum* vaccine development.

STRATEGY:

1. Improve synchronization.
   a. Temperature cycling (timer controlled incubator, 27 h at 37°C, 15 h at 40°C); 40°C causes trophozoites to accumulate.
   b. Alanine lysis of mature parasites, timed after invasion.
   c. Uniform nutrient distribution and one parasite per erythrocyte: suspension cultures (68 rpm rotator):
   d. Preselect uniform erythrocytes: use narrow range of less dense, younger erythrocytes separated by silicone fluids of known densities in density gradient centrifugation.
2. Improve invasion inhibition assay.
   a. Use highly synchronized schizonts (or merozoites later), e.g., to limit the time of exposure of parasites to antibodies.
   b. Preadsorb sera:
      (1) with fumed silica to remove nonspecific toxic factors such as lipid oxidation products.
      (2) with erythrocytes to remove anti-erythrocyte antibodies.
   c. Use suspension culture to slightly separate erythrocytes and give antibodies more time to interact with parasites.
3. Purify viable merozoites: for studying mechanisms of invasion.
   a. Use highly synchronized cultures of schizonts that have been purified on silicone fluid density gradients.
   b. Use inhibitors of signal transduction to stabilize merozoites during purification, remove them to study trigger mechanisms.

CURRENT STATUS:

1. Synchronization: Recently, prepared flasks of highly synchronous schizonts with 50% invasion over 1 hour time, 14 rings/schizont.
2. Invasion assay: Recently, microcultures (100 ul in plates) have been kept in suspension with about 10-fold growth per cycle.

TECHNOLOGICAL BARRIERS: NONE

PLANS:

1. Synchronized parasites should be useful in producing schizont and merozoite antigens (e.g., EBA-175) for monkey vaccination trials.
2. Invasion inhibition assay: begin testing immune sera and antibodies.
3. Viable merozoites: begin production using highly synchronous schizonts. Assay merozoites for viability (invasion) and gradually improve viability by using purification strategies, similar to those for blood platelets, that rely on inhibitors of activation.

RESOURCES:

Need $10,000 centrifuge with heating as well as cooling element to improve temperature control in the range of 22-37°C for the processing of erythrocytes and parasites with silicone fluids that are very temperature sensitive.

COL J. David Haynes/DSN (202) 576-0918/0880

1992 DOD ANTIMALARIAL DRUG DISCOVERY & DEVELOPMENT REVIEW

Division of Experimental Therapeutics
WALTER REED ARMY INSTITUTE OF RESEARCH
United States Army Medical Research & Development Command

Information Papers

Drug-Resistant Malaria Emerges in Cambodia

Spread to Other Parts of the Tropics Feared

By Boyce Rensberger
Washington Post Staff Writer

A new strain of malaria that is resistant to all the standard drugs used to cure the disease has emerged in Cambodia and could spread to other parts of the tropical world, the World Health Organization announced yesterday.

The fear of spread is especially acute, a WHO spokesman said, because the first of about 22,000 soldiers and civilians of a U.N. peacekeeping force are now entering the affected region—a mountainous area on the border with Thailand that has seen years of war between Khmer Rouge guerrillas and government forces.

The U.N. forces, health officials fear, could carry the new strain with them when they return to their homes all over the world. Those who return to tropical countries could carry the parasite, a one-celled protozoan, in their blood. Mosquitoes in the troops' home countries could take in the parasites when they bite the veterans and spread it to others.

Also at risk, according to the WHO, are about 360,000 Cambodian refugees now in Thailand who are expected to return to their homes in the affected region within the next few weeks.

"It's a potentially scary situation," said Dyann Wirth of Harvard University, who heads a malaria research program jointly sponsored by WHO, the World Bank and the United Nations Development Programme.

Wirth said the only treatment available for persons who get the new strain of parasite is a 14-day course of quinine and the antibiotic tetracycline. Whereas the standard drugs are administered in a single dose, the multi-dose regimen is difficult to maintain under Third World conditions. Also, both drugs are in short supply in Cambodia.

"If Cambodia cannot get enough drugs and support for the improvement of health care and training," said Hiroshi Nakajima, director-general of WHO, "there may be a tragedy."

Wirth said the new strain emerged in a region that appears to be a hotbed of malaria parasite evolution. It was there in 1959 or 1960, researchers believe, that the parasite mutated in a way that gave it resistance to chloroquine, a drug that had been used for about 20 years to cure the disease. In ensuing years, chloroquine-resistant strains of the parasite spread throughout the tropics, carried in the blood of infected people.

A number of other drugs have been developed to use where chloroquine fails; but each was eventually defeated by a strain of the parasite. Fansidar, once a highly touted combination of two drugs, lasted for only two or three years before resistance arose and began spreading.

The result has been that different malaria zones around the world have different combinations of parasite strains, including some with no resistance, some that can fend off one drug and some that are invulnerable to several drugs. Currently about 270 million people, most of them in Africa, have malaria and between 1 million and 2 million of them die of the disease each year.

When the latest drug, mefloquine, was developed, researchers deliberately tried to limit its use to cases that were clearly resistant to the other drugs. The theory was that the fewer parasites that encountered the drug, the less the chance resistance would emerge. Yet mefloquin remained unchallenged for only six or seven years.

Wirth said the emergence in Cambodia of a parasite strain that can resist not only all other drugs but mefloquine as well has now been confirmed both in patients and in laboratory tests. She said there is suspicion that some parasites have evolved a generalized form of resistance to foreign chemicals—a mechanism that enables the cells to pump out noxious chemicals.

The affected area of Cambodia is rich enough in gems and timber that a number of people have immigrated to the region in hopes of making a living, despite the fact the ground holds land mines left over from years of wars.

"Though mines are killing perhaps two to four people a day," said Jean-Paul Menu, WHO's special health envoy to Cambodia, "malaria is killing very many more."

Am. J. Trop. Med. Hyg., 45(1), 1991, pp. 98–111

CHARACTERISTICS OF MULTIDRUG RESISTANCE IN *PLASMODIUM* AND *LEISHMANIA*: DETECTION OF P-GLYCOPROTEIN-LIKE COMPONENTS

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Abstract. Multidrug-resistance (MDR) in neoplastic cells is frequently characterized by the overexpression of P-glycoprotein (PGP), a 170 kDa transmembrane glycoprotein that binds multiple cytotoxic drugs as well as calcium channel antagonists. Chloroquine resistance in *Plasmodium falciparum* appears to be analogous to MDR in neoplastic cells, where the induction of resistance with one drug confers resistance to other structurally and functionally unrelated drugs. To test the hypothesis that chloroquine resistance in *P. falciparum* and antimony resistance in *Leishmania* is mediated by a similar mechanism of MDR in mammalian neoplastic cells, a PGP-specific monoclonal antibody (C219) was used to determine the presence of PGP genes in resistant and sensitive *Plasmodium* and *Leishmania* parasites by indirect immunofluorescence assays and Western blotting procedures. These PGP-like components were detected in both drug-sensitive and -resistant *Plasmodium* and *Leishmania* cells. A 40–42 kDa component was observed to be greater in a chloroquine-resistant *P. berghei* (C line) than in a chloroquine-susceptible P line. Differences observed between Pentostam-resistant and -sensitive *Leishmania* promastigote clones and isolates included the increased expression of 96–106 and 23–25 kDa peptides in drug-resistant *L. enriettii*, and increased amounts of two different peptides in two drug-resistant *L. panamensis* clones (i.e., 96–106 and 43–45 kDa in WR-746-CL4, and 53 and 23–25 kDa in WR-746-CL6). Interestingly, C219 detected a peptide of the same molecular weight (170 kDa) in amastigotes as in MDR KB carcinoma cells (KB-V1).

Comparative indirect immunofluorescent studies suggested that a correlation existed between the degree of antimony susceptibility and the concentration of the moiety recognized by C219 in two *L. panamensis* clones. Binding of the C219 monoclonal antibody to the PGP-like component of *Leishmania* was blocked by Pentostam, while the binding of C219 to multiple-drug resistant KB-V1 PGP was not inhibited by Pentostam, regardless of the PGP concentration. This suggests some degree of specificity in the binding of Pentostam to the *Leishmania* PGP-like components. In addition, these studies have demonstrated that drug-sensitive *Leishmania* accumulate two to five times more ^{125}Sb-Pentostam than resistant clones.

Information Paper

Department of Medicine
U.S. Army Medical Component
AFRIMS

21 MAY 1992

SUBJECT: Clinical Significance of Drug Resistant Malaria in Indochina and Recent Antimalarial Drug Trials at AFRIMS

OBJECTIVE: To review the clincal problems currently being experienced in Southeast Asia, to summarize the recently completed antimalarial drug prophylaxis and treatment trials, and to underscore the urgent need for continued antimalarial development and drug trials.

CURRENT STATUS:

1. Lack of financial incentives for antimalarial drug development
2. Enormous morbidity and mortality and potential risk to U.S. troops
3. Department of Medicine, AFRIMS, research goals
4. Emergence of multidrug antimalarial resistance in Thailand
5. Current clinical problems being encountered in Thailand
6. Worldwide spread of antimalarial drug resistance
7. Recent AFRIMS antimalarial prophylaxis trials
8. Recent AFRIMS antimalarial treatment trials
9. Urgent need for continued antimalarial development and testing

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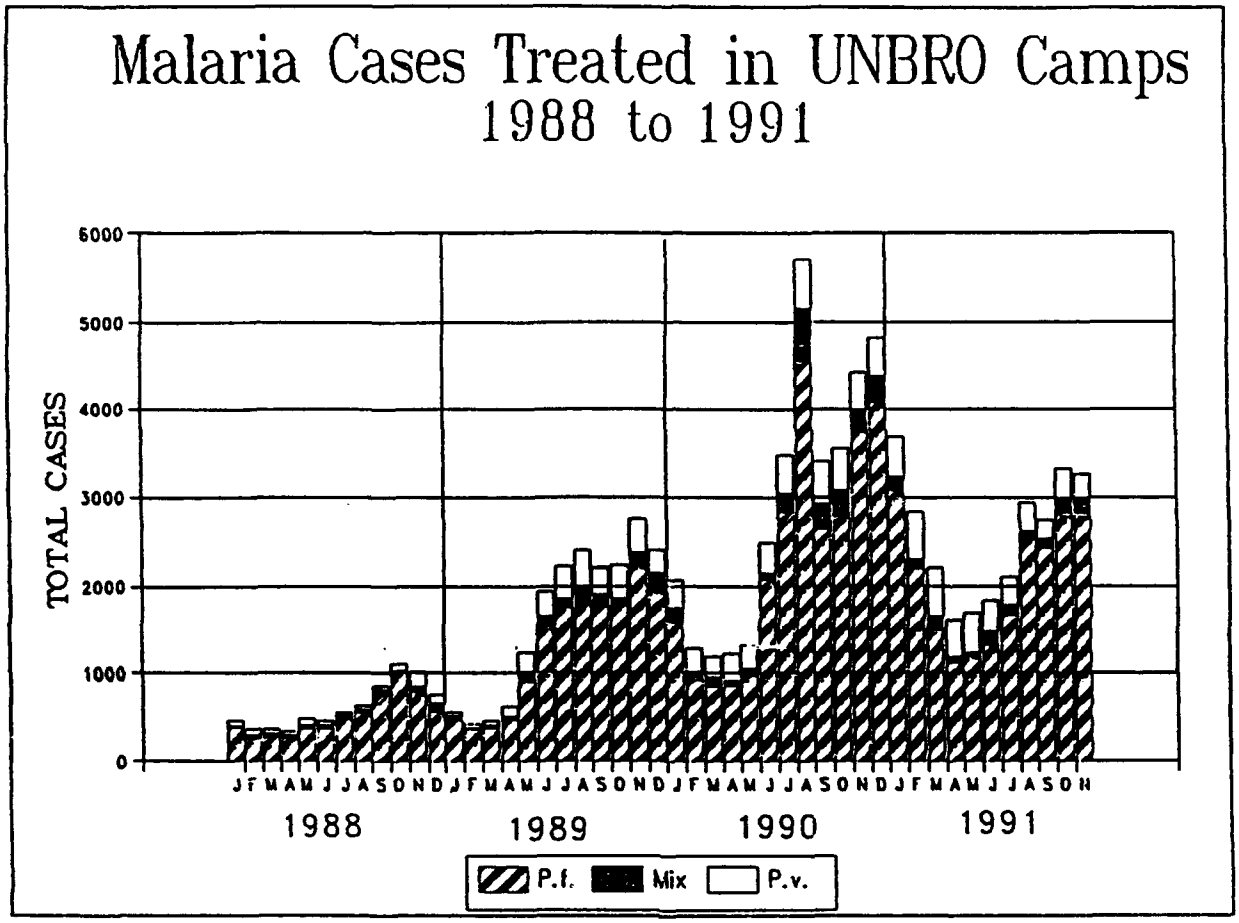


History of Antimalarial Use on the Thai-Cambodian Border:

1981 : Chloroquine and Sulfadoxine-Pyrimethamine changed to Quinine plus Tetracycline for seven days

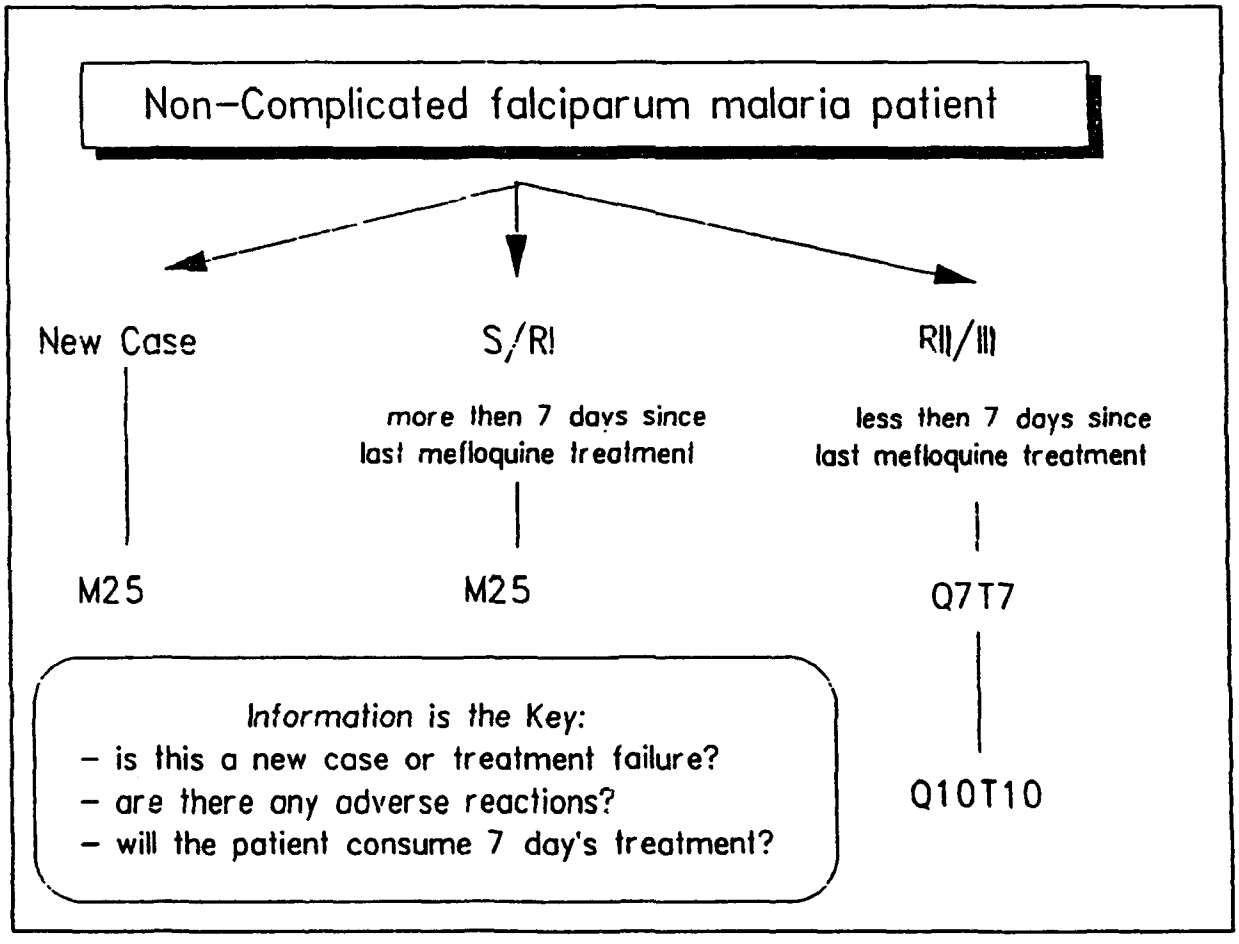
1984 : Mefloquine-sulfadoxine-pyrimethamine plus primaquine
-1985 Q7T7 remains backup

1989 : Discontinue use of Primaquine

1990 : Switch from MSP to mefloquine alone (15 mg/kg)
Q7T7 remains backup

Ongoing Resistance Studies:

Camp	Dose	Cure Rate	Follow-up
O'Trao	M15	86%	28 Day
Sok Sann	"	79	"
Site B	M15	66	"
	M25	88	"
	M15	48	42 Day
	M25	69	"
Site 8	M15	40	"
	M25	44	"



Information Paper

Department of Medicinal Chemistry
Division of Experimental Therapeutics
Walter Reed Army Institute of Research

21 May 1992

SUBJECT: Inhibition of Malarial DNA Polymerase Alpha

OBJECTIVES: To identify new antimalarial drugs that selectively inhibit P. falciparum DNA polymerase alpha.

STRATEGY:

1. Purify and characterize P. falciparum DNA polymerase alpha
2. Identify plant extracts having potent, selective inhibitory activity toward the P. falciparum DNA polymerase alpha but not toward human DNA polymerase alpha or beta.
3. Fractionate the extracts containing putative inhibitors and obtain the active principles by bioassay-guided fractionation.
4. Elucidate the structures of the isolated principles.
5. Obtain the active principals in sufficient quantities for development.

CURRENT STATUS:

1. Unable to obtain sufficient parasite to isolate the enzyme in the quantities needed, have undertaken the cloning of P. falciparum polymerase alpha. Have obtained and sequenced a number of clones that encompass approximately 2000 nucleotides that encodes a major portion of P. falciparum DNA polymerase alpha. Have identified the invariant regions characteristic of all know polymerases alpha and one intervening sequence that has been demonstrated for other enzymes of this type. Have prepared oligonucleotide proble molecules that allows extension of the sequence in both the 3' and 5' direction. Anticipate completion of the cloning and expressing of the enzyme within a few months, thus making sufficient P. falciparum DNA polymerase alpha available.
2. Identified 4 extracts that have demonstrated moderate inhibition of human DNA polymerase alpha and also inhibited the growth of P. falciparum (both D6 and W2 clones) in vitro. The extracts were also non-cytotoxic toward cultured Vero cells. Three fractionation steps guided by in vitro bioassay have been completed and the antimalarial activity has been enhanced dramatically relative to the initial extracts. The fractionation work is continuing to permit isolation and structure elucidation of the active principles.

TECHNOLOGICAL BARRIERS:

1. Availability of sufficient parasites from which to extract the enzyme.

PLANS (to overcome barriers):

1. Clone and express the enzyme.

RESOURCES:

1. Contract terminates 14 September 1992 (research effort completed 14 April 1992).

2. Renewal proposal awaiting funding (160K for FY-93).

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INFORMATION PAPER

Division of Experimental Therapeutics
Walter Reed Army Institute of Research

21 May 92

SUBJECT: Characterization of MDR Phenotypes in Falciparum Malaria

OBJECTIVE: Perform *in vitro* studies to determine mechanisms of resistance to different antimalrial drugs in malaria parasites.

STRATEGY: Obtain various parasite clones and strains from different geographic areas and perform quantitative drug interaction studies.

CURRENT STATUS: The MDR phenotype observed in falciparum shares similarities with the MDR phenotype in human neoplasias: pleiotropic drug resistance; the presence of an energy dependent p-glycoprotein mediating drug efflux; and the reversal of resistance by sub-inhibitory concentrations by calcium antagonists (verapamil) and the phenathiozines (chlorpromazine). The latter are drugs that in combination with the antimalarial drug potentiate its effect on the resistant parasite. Studies of the p-glycoprotein gene, *pfmdr*1, had suggested that mutations of specific amino acid residues may be associated with chloroquine resistance. Additional data has implied that the p-glycoprotein was not sufficient to mediate the chloroquine-resistance phenotype and that chloroquine resistance was multigenic. Other findings had described the amplification of *pfmdr*1 in mefloquine-resistant isolates and clones found only in Thailand. In order to ascertain the extent of the involvement of the p-glycoprotein and other candidate genes/mechanisms in drug resistance, we developed an approach to identify drug resistance phenotypes. The basis of this approach involved *in vitro* studies with reversal modulators. We have identified three distinct drug reversal phenotypes based upon the geographic origin of the parasite and the reversal modulators.

1. The first phenotype, chloroquine-resistance, was found to be similar throughout the world.

2. The second involved the reversal of mefloquine-resistance and was limited to sub-Saharan Africa.

3. The last phenotype also involved mefloquine resistance but was restricted to Southeast Asia and was noted to be distinct from the African phenotype. The description of these phenotypes will further the characterization of the genetics of MDR and assist in the lead-directed synthesis of new compounds to circumvent these resistance mechanisms.

PLANS: The description of these phenotypes will further the characterization of the genetics of MDR and assist in the lead-directed synthesis of new compounds to circumvent these resistance mechanisms.

RESOURCES:

1. Highly dependent on availability of strains from the AFRIMS, Peace Corps & State
2. Investigations limited by funding

POC: LTC Wilbur K. Milhous, PhD WRAIR Div-ET 301-427-5029

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Table 1. Table of reversal phenotypes based on reversal modulators that decrease parasite resistance to the antimalarial drug listed at the top of each respective column.

	Reversal Phenotype I	Reversal Phenotype II			Reversal Phenotype III		
Origin of Parasite Isolates	Chloroquine and Quinine	Mefloquine	Halofantrine	Artemisinin	Mefloquine	Halofantrine	Artemisinin
West Africa	Verapamil Chlorpromazine Desipramine Amitriptyline Prozac Cyproheptadine Promethazine	Penfluridol[1]	Penfluridol[1]	Penfluridol[1]			
Southeast Asia	Verapamil Chlorpromazine Desipramine Amitriptyline Prozac Cyproheptadine Promethazine				Penfluridol Verapamil Chlorpromazine Desipramine Amitriptyline	Penfluridol Verapamil Chlorpromazine Desipramine	Penfluridol[2]
South America	Verapamil Chlorpromazine Desipramine	NA[3]	NA[3]	NA[3]	NA[3]	NA[3]	NA[3]

[1]Penfluridol is the only reversal modifier found to decrease resistance to mefloquine, halofantrine and artemisinin in West Africa.

[2]Reversal of artemisinin resistance by chlorpromazine and desipramine is suspected but not confirmed.

[3]There is no known resistance to mefloquine, halofantrine and artemisinin in South American *P. falciparum* parasites.

Information Paper

Division of Experimental Therapeutics
Walter Reed Army Institute of Reserarch

21 May 1992

SUBJECT: Organellar RNA polymerase of P. falciparum

OBJECTIVES: To determine the sequence and analyze expression of genes encoding the two largest subunits of the organellar RNA polymerase (RNAP), and to explore the potential of this enzyme as a new target for chemotherapy.

STRATEGY:

1. Determine rpoB and rpoC gene sequences by sequencing overlapping clones to be isolated from a shotgun library.
2. Analyze expression of the RNAP genes in erythrocytic parasites using Northern blots and RNAase protection assays.
3. Use ^{3}H-hypoxanthine assay to determine susceptibility of various drug resistant parasites to the RNAP inhibitor rifampicin.
4. Test rifampicin analogs for antimalarial activity.

CURRENT STATUS: Cloning. The entire rpoB sequence has been determined. It encodes a polypeptide 1024 amino acids in length which is approximately 30% identical to the corresponding bacterial and chloroplast subunits. Important functional domains of the bacterial subunit involved in substrate binding, holoenzyme assembly, and rifampicin resistance appear to be conserved between species. A phylogenetic comparison of the bacterial, chloroplast, and malarial beta subunits is in progress (R.J.M. Wilson, NIMR, London). The 5' one-third of the malarial rpoC gene encoding the beta prime subunit was sequenced previously. Probes to the 3' end of the gene have been prepared and will be used to isolate the remainder of this gene from a shotgun library of the 35 kb DNA.

Expression. Previous studies have shown that these genes are transcribed in erythrocytic parasites, probably as polycistronic transcripts encompassing both the rpoB and rpoC genes. RNAase protection experiments to map transcripts of the rpoB gene are underway, and will be extended to the rpoC gene once the gene sequence is determined.

Rifampicin assays. In preliminary experiments six P. falciparum clones or isolates were tested for rifampicin sensitivity. All of the parasites had IC50s below 3000 ng/ml, well within the range of serum concentrations achieved during tuberculosis chemotherapy. In addition there appears to be a correlation between mefloquine resistance and sensitivity to rifampicin.

TECHNOLOGICAL BARRIERS:

PLANS: Work to be completed by the end of NRC Fellowship - October '93.

RESOURCES: The Division does not possess a critical mass of professional and technical expertise in molecular biology pursue these types of studies. In addition, progress has been severely limited by the lack of laboratory space dedicated to molecular biology. At present, molecular work is conducted in space borrowed in two laboratories. Overcrowding is severe with equipment, reagents, and personel distributed between the two laboratories. The situation has been exacerbated by additional demands on personel and space resulting from the Leishmaniasis crisis. Plans for converting an office into a laboratory have been prepared but not yet implemented. Completion of the laboratory is essential for development of a molecular biology program to assist the Division's drug discovery efforts.

POC: Malcolm J. Gardner, PhD, NRC Fellow, 301-427-5304

Information Paper

Department of Pharmacology
Division of Experimental Therapeutics
Walter Reed Army Institute of Research

21 May 1992

SUBJECT: Applications of Molecular Modeling to Drug Discovery & Design

OBJECTIVE: To design new antiparasitic drugs with higher activity and fewer toxic side effects from the 3-dimensional structure of existing antiparasitic agents and their receptor sites.

STRATEGY:

1. Compilation of 3-dimensional structures of existing antiparasitic drugs
   a. Determine 3-dimensional structure using x-ray crystallography
   b. Use previously determined structures from the Cambridge Structural Database
   c. Estimate 3-dimensional structures using the SYBYL molecular modeling program
2. Relate 3-dimensional structure and electronic properties to activity
   a. Determine electronic structure of antiparasitic drugs using SYBYL and other electronic modeling programs
   b. Determine relative activity of families of antiparasitic agents which have the same mechanism of action
   c. Make model of receptor site from the known structural and electronic features of the antiparasitic agents and their relative activity
   d. Use macromolecular crystallography to determine actual structure of receptor site when receptor site is known
3. Design new antiparasitic candidate drugs
   a. Using model of receptor site or actual receptor site, design antiparasitic agents with the structural and electronic features which best fit into and interact with the site

CURRENT STATUS:

1. Established the x-ray crystal structure of natural and synthetic amino alcohol antimalarial agents including mefloquine, halofantrine, the *threo* analog of mefloquine, enpiroline, WR 194,965, 9-epiquinine, and 9-epiquinidine to assist in modeling a receptor.

2. Established the relative antimalarial activity of quinine, quinidine, 9-epiquinine, and 9-epiquinidine showing that the 9-epi alkaloids are weakly active against *Plasmodium falciparum in vitro* in comparison to quinine and quinidine.

3. A comparison of the 3-dimensional structures of quinine and quinidine with the 3-dimensional structures of the 9-epi alkaloids reveals that the relative spatial positioning of the amine and alcohol groups is the most likely determinant for their reduced antimalarial activity.

4. Determined the absolute configuration of (+)- and (-)-mefloquine hydrochloride by x-ray crystallography and showed that the more active enantiomer of mefloquine has the same absolute configuration as quinidine and the less active mefloquine enantiomer has the same absolute configuration as quinine. Quinidine is more active than quinine against *P. falciparum*.

5. Determined the relative antimalarial activity of enantiomeric pairs of synthetic amino alcohol antimalarial agents showing that the enantiomers of mefloquine and its *threo* isomer to exhibit the most difference in antimalarial activity. The enantiomers of halofantrine were equally activity.

6. A 3-dimensional model of the site of action of amino alcohol antimalarial agents is being created using the information obtained in the above studies.

TECHNOLOGICAL BARRIERS:

1. Insufficient computer hardware for fully implementing molecular modeling program. With present computer cannot dock molecules into receptor site, an invaluable feature for determining if a compound can fit into a receptor site.

PLANS: (to overcome barriers)

1. Upgrade computer hardware

RESOURCES:

1. NRC Fellow with expertise in modeling the electronic structure of pharmaceutical agents

2. Funds for upgrade of computer hardware, estimated cost $65,000

Jean M. Karle, Ph.D./301-427-5177

Publications:

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MALARIA RESEARCH REVIEW

Division of Experimental Therapeutics
Walter Reed Army Institute of Research

May 21, 1992

SUBJECT:

Traditional Medicine and Natural Products in Drug Discovery and Design

OBJECTIVE: To identify, isolate and characterize new chemotypes for the treatment of drug-resistant malaria based on leads from traditional medicine and naturally occurring molecules. To delineate the compounds and structural moiety responsible for the antimalarial activity of Picralima nitida.

STRATEGY:

1. Since nearly all antimalarial drugs in current use were developed from plants used in traditional medicine, or modification of naturally occurring molecules, the basic approach is to examine extracts of medicinal plants for activity against W-2 and D-6 clones of Plasmodium falciparum. The most active extracts are further fractionated and the active compound(s) isolated by bioassay guided chromatographic separation.
2. The active compounds will be tested in vivo to determine their activity against P. berghei and to obtain information on their toxicity.
3. The structures of the isolated compounds established from their spectra data (including MS, FT-IR, ^{1}H and ^{13}C nuclear magnetic resonance) will be used as templates for rational design and optimization of the naturally occurring molecules.

CURRENT STATUS:

Six compounds WR276491, WR019046, WR279180, WR279181, WR279183, and WR279189 isolated from the fruits of west African plant Picralima nitida, were identified as potential new antimalarial agents. The alkaloids gave IC_{50} values of 0.01 - 0.06 µg/ml when tested in vitro against W-2 and D-6 clones of Plasmodium falciparum. The in vitro antimalarial activity of these alkaloids were found to be comparable to those of the clinical antimalarial chloroquine and quinine. The Picralima alkaloids represent an entirely new antimalarial chemotype with possible pharmacokinetic advantages over the existing drugs. Some of the compounds have also been shown to be active in vitro against Leishmania. Alstonine, one of the most active isolates, was found to be non-toxic to human lymphocyte CEM T_4 cells at up to 100 µg/ml.

Thirty-six compounds with related chemical structures have been examined by in vitro bioassay to establish a tentative structure/activity mapping of the class. Four main chemical clusters were recognized as possible templates for hemi-synthetic optimization and design of compounds with improved activity. The heteroyohimbines and 2-acylindoles appear to be the delimitative class among indole alkaloids as potential chemotherapeutic group for the treatment of drug-resistant malaria.

TECHNOLOGICAL BARRIERS:

Although synthetic routes leading to this class of compounds are well known, these alkaloids are not commercially available and must be obtained (for now) from their natural sources. The yield of the active compounds in the plants are extremely low, making it difficult to obtain enough material for in vivo antimalarial studies, as well as determining their pharmacokinetics and toxicity. Facilities are not readily available for large scale extraction and isolation of the active compounds.

PLANS (to overcome barriers)

A grant has been obtained from the World Health Organization,which provides for a pilot scale extraction and separation of Picralima nitida at the University of Nigeria Nsukka, to obtain gram quantities of the pure compounds for more elaborate biological studies. A collaborative arrangement is already in place between WRAIR and the University of Nigeria Nsukka.

Preparative scale extraction and isolation of the active compounds are expected to be completed by the end of 1992. The in vivo studies will be initiated early in 1993.

RESOURCES

The project is presently supported through the NRC Associateship Program, and funds from the World Health Organization. A grant application to US-AID has been favorably reviewed and may receive funding from August 1992. Logistics assistance may be required to an effective coordination of the research collaboration between WRAIR and the University of Nigeria.

Dr. Maurice M. Iwu

INFORMATION PAPER

Division of Experimental Therapeutics **21 May 92**
Walter Reed Army Institute of Research

SUBJECT: Evaluation WR269854 as Resistance Modulator
OBJECTIVE: Design and development a novel reversal modulator to circumvent chloroquine resistance of *P.falciparum* and understand the possible role of p.glycoprotein in chloroquine resistance.

STRATEGY: The discovery that the chloroquine resistant *P.falciparum* parasites release chloroquine 40 to 50 times more rapidly than the susceptible parasites led the present approach to search for new agent to be able to reverse this process. The reduced accumulation of chloroquine has been associated with active efflux of drug from resistant parasites. The increased drug efflux is an energy dependent event and may be mediated by parasite membrane bound p-glycoprotein. The role of p-glycoprotein in chloroquine resistant *P.falciparum* has yet been understood clearly, however, like MDR-tumor cells, it appears overexpression of p.glycoprotein in falciparum malaria may render the parasites resistant to chloroquine and other 4-aminoquinolines. The crucial role of p.glycoprotein has identified itself an important target site for designing prospective chloroquine resistant reversal modulators.

CURRENT STATUS: A few novel chemical agent have been synthesized of which the best active one is WR269854, a pyrrolidino alkane amine compound. This compound, a non 4-aminoquinoline may mimic chloroquine thus, at least hypothetically, can compete with the chloroquine to bind at the receptor sites of p.glycoprotein. WR268954 lacks significant intrinsic antimalarial activity. In-vitro, WR268954 reverses chloroquine resistance of *P.falciparum* from different geographical origins and it appears WR268954 more active when compared to known chloroquine resistant modulator verapamil. Initial in vivo studies indicate WR268954 in combination with chloroquine suppresses parasitemia substantially in chloroquine resistant *P.falciparum* infected Aotus monkey.

TECHNOLOGICAL BARRIERS:

1. Aotus monkey resource limits extensive evaluation
2. Availability of monoclonal antibody specific for p-170 glycoprotein.

PLANS:

1. WR268954 is a lead directed synthetic chloroquine resistant modulator. Detail studies are needed and efforts are going on to test degree of parasitemia inhibition, recrudescence or toxicity in Aotus monkey with the combination of chloroquine and WR268954.
2. An attempt to identify the presence of p.glycoprotein in *P. falciparum* with a monoclonal antibody specific for p-170 glycoprotein in MDR-neoplastic cells will be undertaken which can definitely be able to describe the possible role of p.glycoprotein in chloroquine resistance and vis-a-vis mechanism of reversal of chloroquine resistance in *P. falciparum*.
3. To study the effect of compound WR268954 on the accumulation and release of radiolabelled chloroquine in chloroquine resistant and sensitive *P. falciparum* in-vitro.

RESOURCES:

1. Laboratory space has been limited because of Desert Storm Leishmaniasis Project.
2. Will require additional $7-10K for radiolabelled chloroquine.

POC: DIBYENDU DE, PhD, NRC Fellow, WRAIR Div-ET 301-427-5029

Information Paper

Department of Medicinal Chemistry
Division of Experimental Therapeutics
Walter Reed Army Institute of Research

21 May 1992

SUBJECT: Artemisinin Analogs - Synthetic Effort

OBJECTIVES: To design and synthesize analogs of artemisinin that have enhanced antimalarial potency, improved water solubility and stability, oral activity, and/or longer duration of activity.

STRATEGY:

1. In-house synthetic effort

   a. Artemisitene Derivatives - Dr. Nancy Roth

   b. Dihydroartemisinin Derivatives - Dr. A. J. Lin and Dr. Mai Van Tri

2. Extramural (contract) effort

   a. Structural modifications of Artemisinin - Contract DAMD17-91-C-1099 (Dr. Mitchell Avery, University of North Dakota)

CURRENT STATUS

1. In-house

   a. Established that modification of the lactone ring (D ring) affects biological activity.

   b. Converted artemisitene into 9-substituted artemisinin derivatives

   c. Converted dihydroartemisinic acid to artemisinin

   d. Synthesized analogs of dihydroartemisinin

   e. Showed that artelinic acid (sodium artelinate) is water soluble, stable and has good antimalarial activity

2. Contract

   a. Developed total synthesis of artemisinin

   b. Synthesized rings A and D structural modifications

c. Shown that the artemisinin antimalarial pharmacaphore is highly specific and requires the full tetracyclic ring system for good activity

d. Done SAR and modeling studies.

TECHNOLOGICAL BARRIERS:

1. Limited synthetic effort. No new areas being investigated.

PLANS (to overcome barriers):

1. Initiate new synthetic effort to investigate potentially new antimalarial compounds.

RESOURCES:

1. Need stable funding to support synthetic efforts.

2. Funding for NRC Fellow

3. Funding to initiate investigation of potentially new antimalarial compounds.

Dr. Robert R. Engle
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INFORMATION PAPER

Division of Experimental Therapeutics 21 May 1992
Organic Chemistry Section

Subject: The evaluation of the antimalarial candidate artelinic acid and its analogs.

Objective: To develop water-soluble artemisinin derivatives with enhanced antimalarial activity and to explore transdermal delivery of artemisinin analogs for both prophylactic and curative purposes.

Strategy:

1. Design and prepare improved water-soluble artemisinin derivatives.
2. Investigate oral and parenteral administration of artelinic acid (U.S. Patent 4,791,135; assigned to Army) and related compounds.
3. Investigate transdermal administration of artemisinin class of compounds.

Current status:

1. The synthesis of new compounds related to artelinic acid by the Organic Chemistry Section is in progress.
2. Transdermal evaluation in mice of 8 artemisinin class of compounds is being completed at University of Miami. Results are very encouraging.
3. A U.S. patent application, related to transdermal artemisinin administration, has been filed.
4. Artelinic acid patent is expected to be licensed near the end of May 1992 on non-exclusive basis.

Technological barriers:

1. Organic Chemistry Section lacks ability to do bioavailability and pharmacokinetic studies in animals on transdermally administered artemisinin-related compounds
2. Similarly, we lack means to test for efficacy in higher animals.
3. Present transdermal formulation need fine tuning.

Plans:

1. To investigate the impact of varied dosing regimens in mice.
2. To study use of membranes for transdermal patches. (Perhaps drug administration and absorption should be slowed.)
3. To use limited powers of persuasion to have transdermal experiments performed in animals other than mice.

Resources:

1. Perhaps a contract should be let to perform required animal studies.

Daniel. L. Klayman, Ph.D.
Head, Organic Chemistry Section

Information Paper: Malaria Program Review

Department of Pharmacology
Division of Experimental Therapeutics
Walter Reed Army Institute of Research

21 May 92

Subject: Preclinical Metabolism and Pharmacokinetic Studies to Support Development of Arteether and Artemisinin Analogs

Objectives:

1. Gain needed information regarding metabolism, disposition and pharmacokinetics of new artemisinin antimalarial drugs.

2. To discover important antimalarial and toxic properties of identified metabolites.

Strategy:

1. Methods development. Apply techniques using HPLC with detection systems including UV, fluorescent, electrochemical, and other means of detection as well as mass spectroscopy in combination with gas chromatography or HPLC to unambiguously identify metabolites of interest.

2. Perform initial in vivo and in vitro experiments to identify the primary metabolism pattern of artemisinin drugs - especially arteether - using model test systems.
a. Microsomes
b. Subcellular metabolic systems
c. Isolated hepatocyte cells and isolated perfused liver
d. *In vivo* animal metabolism
e. Pharmacokinetics of parent drug (and metabolites if available) in rats, dogs or other species of interest.

3. Use model metabolism systems to discover metabolites to test for antimalarial activity in bioassay *in vitro* systems.

4. Use information gained in basic metabolism studies to support and direct contract toxicology and metabolism and pharmacokinetic studies under contract and ultimately to obtain information to support clinical field studies.

Current Status:

1. After evaluation of several analytic test systems, developed and validated an HPLC reductive electrochemical detection system using automated sample input for analysis of arteether and its primary metabolite, DQHS, in biologic fluids.

2. With modifications of method noted above, have developed and are validating methods for detection of arteether, artemether, DQHS, artelinic acid and artesunate in biologic fluids.

3. a. Established metabolic profile of arteether in liver microsomes and isolated perfused liver. Performed initial disposition and bioavailability studies of arteether in the rat and dog.

b. Demonstrated rapid hepatic clearance of arteether with generation of a primary metabolite, dihydroqinghaosu (DQHS), in in vitro systems and confirmed in in vivo dog studies. Finding that

the endoperoxide moiety was well preserved in several metabolites implying preserved efficacy of several metabolites.

c. The arteether in sesame oil preparation was shown to have an erratic, slow but complete absorption from the intramuscular site as shown by ^{14}C arteether studies in rats. Radioactive studies show persistent radioactivity detected in blood implying long lived metabolites. Drug is extensively metabolized with virtually no parent drug excreted in either urine or feces. The drug is eliminated in both urine (35-40%) and feces (45-50%). There is no apparent tissue specific accumulation of the drug by radiochemical studies.

d. A fatal neurotoxicity syndrome was demonstrated in two species with at least two QHS analogues. Related to this clinical neurotoxicity is localized CNS lesion which is dose and time related and thought to be associated with an unknown long-lived metabolite.

4. Present activities include an active pursuit in pilot studies of several clinical findings that could be used as sentinel indicators in future animal and clinical studies. We are now using an in vivo rat model specifically developed for the purpose of outlining the toxicokinetics of this neurotoxicity.

Technologic barriers:

Needed studies to support further development of drug are critically constrained by inadequate personnel resources. Requirement for dose ranging toxicokinetic studies include microcomputer hardware and software, additional capillary electrophoresis capability will probably be needed, and alternative (more field expedient) analytic methods need to be developed.

Plans:

1. Personnel augmentation for more intensive LC-mass spectroscopy support of animal studies to determine toxic metabolite.

2. Develop field expedient analytic method for arteether and other artemesinin analogs.

3. Determine dose toxicity relationship and identification of arteether metabolite responsible for CNS toxicity.

Resources:

1. Stabilized personnel status of only full time Ph.D. pharmacologists in metabolism section.

2. NRC Fellow to continue studies in arteether/DQHS metabolism.

3. Recruiting action for Ph.D. pharmacologist for Medical Service Corps position.

4. Funding for toxicology and metabolism studies noted above.

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Information Paper

Division of Experimental Therapeutics **21 May 92**
Walter Reed Army Institute of Research

SUBJECT: Evaluation of Synthetic Peroxides

OBJECTIVE: To synthesize, evaluate and develop effective synthetic peroxide antimalarial drugs for prophylaxis, and treatment of both early and complicated malaria.

STRATEGY: The discovery of qinghaosu (artemisinin) as a potent antimalarial drug has sparked an intense search for new compounds sharing artemisinin's activity, but that would be more economical to synthesize with improved pharmacologic properties. Two different classes of compounds, referred to as synthetic peroxides because they incorporate an endoperoxide bridge that is necessary for antimalarial activity, have been investigated and include members with clinical potential. All share a relatively simple synthesis, which would be an advantage over artemisinin or semisynthetic derivatives which are more expensive to extract from the plant and make derivatives.

CURRENT STATUS: Trioxanes; Over 200 trioxanes have been synthesized by Dr. Charles Jefford of the University of Geneva and sent to the WRAIR under WHO auspices for *in vitro* antimalarial testing. Several have demonstrated *in vitro* activity greater than artemisinin. The compounds were sent to us "blind" and a co-development agreement will probably be pursued through a private pharmaceutical company. Trioxanes with distinct chemical structures have been synthesized by Dr. Gary Posner of Johns Hopkins University, and sent to the WRAIR for antimalarial evaluation. Several have shown greater *in vitro* potency than artemisinin. Five have shown curative activity at 640 mg/kg in a multiple dose mouse model experiment.

Tetraoxanes; Several tetraoxanes have been synthesized by Dr. Jonathan Vennerstrom of the University of Nebraska and sent to the WRAIR as gifts. They have curative activity in the single dose *mouse/P. berghei* model "Rane test". Artemisinin and derivatives prolong survival in this model but seldom produce cures. Three have been found with curative activities less than 640 mg/kg. The most active, WR148999 is as active as artemisinin *in vitro.* Artemisinin and WR148999 were compared in a multiple dose experiment in the mouse designed to estimate the therapeutic index by evaluating efficacy and toxicity in the same experiment. Results revealed similar efficacy of the two, and no toxic deaths up to six grams per Kg. A mouse model test to evaluate oral absorption of this compound, and to test it against artemisinin resistant parasites is in progress.

TECHNOLOGICAL BARRIERS:

1. Uncertainties about bioavailibility in primate animal models and in humans
2. Uncertainties about toxicity.
3. Insufficient synthetic capability for the number of compounds required for definitive SAR studies and to make new compounds as specific problems of bioavalibility and toxicity arise.

PLANS: Tetraoxanes; An experiment to test the efficacy of WR148999 will be starting soon in the *Aotus* primate model. Dr. Vennerstrom is continuing to synthesize new tetraoxanes for a structure activity relationship study and has recently sent seven more for antimalarial testing. Our goals are to improve potency through further SAR, and to answer questions of bioavailability and toxicity.

Trioxanes; Experiments to asses the efficacy of WR279138 and WR279137, the most active of Dr. Posner's compounds, in the *Aotus* model will be starting soon. As with the tetraoxanes, questions of bioavailability and toxicity need to be answered before any synthetic peroxides would be selected for advanced development.

General; All the synthetic peroxides submitted to WRAIR are poorly soluble in water. Two strategies are being pursued; One is to make water soluble derivatives of the most active compounds. Another option migyht be o use the new formulation technology of "molecular encapsulation" to render insoluble compounds soluble in water.

RESOURCES: Medicinal chemists with the expertise for SAR exist and are willing to work with the WRAIR as outlined above. It is noteworthy that all compounds submitted so far have been as gifts with no Army financial support. The Division of Experimental Therapeutics has the resources to evaluate drugs received. Both Dr. Posner and Dr. Vennerstrom have submitted grants for funding to the USAMRDC.

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Information Paper

Division of Experimental Therapeutics
Walter Reed Army Institute of Research

13 May 92

SUBJECT: Evaluation of Bisquinolines as antimalarial drugs

OBJECTIVE: To synthesize, and evaluate and develop new 4-Amino quinolines as chloroquine/mefloquine replacements for the prophylaxis and treatment of malaria.

STRATEGY: Recent discoveries at the WRAIR and collaborating institutions have revealed that the mechanism of action of chloroquine is distinct from the mechanism of resistance. Based on observations that several bisquinolines such as piperaquine posses notable activity against chloroquine resistant malaria, thirteen novel bisquinolines were synthesized by Dr. Jonathan Vennerstrom, a former NRC fellow at WRAIR, and currently at the University of Nebraska, and sent to the WRAIR as gifts. The bisquinoline class of compounds has a high potential for yielding a clinical candidate effective against chloroquine resistant malaria.

CURRENT STATUS: Twelve of the bisquinolines submitted by Dr. Vennerstrom had a significantly lower resistance index than did chloroquine. Seven of the compounds were more active than chloroquine in vitro against chloroquine sensitive parasites and were curative against *P. berghei* at a dose of 640 mg/kg in the mouse model. The most active compound, WR268668 was selected for further testing in the multiple dose rodent model "Thompson test". In an oral/subcutaneous test no toxicity was observed up to three grams per Kg. Good oral absorption was demonstrated by a similar ED_{50} for both routes. The compound was curative at doses below 40mg/kg. *In vitro* assessment of WR268668 revealed virtually no cross resistance with highly chloroquine resistant clones of *P.falciparum*. In contrast to the highly favorable results in the mouse model and *in vitro* test systems, WR268668 when given orally to *Aotus* infected with chloroquine resistant strain Vietnam Smith was not curative even at high doses. This may be the result of poor absorption in the *Aotus* compared to the mouse gastrointestinal system.

TECHNOLOGICAL BARRIERS:

1. Uncertainties about bioavailability in primate animal models compared to rodents and implications for humans.
2. Uncertainties about amount of cross resistance.
3. Insufficient synthetic capability for the number of compounds required for definitive SAR studies and to make new compounds as specific problems of bioavalibility and toxicity arise.

PLANS: WR268668 will be tested in *Aotus* infected with a chloroquine sensitive strain of parasite. If the drug cures an infection with chloroquine sensitive parasites but not chloroquine resistant parasites then we will abandon evaluation of WR268668 and pursue SAR in an attempt to circumvent resistance. New compounds are being synthesized to improve oral absorption.

RESOURCES: A medicinal chemist with the expertise for SAR is willing to work with the WRAIR as outlined above. <u>All compounds submitted so far have been as gifts with no Army financial support.</u> The division of Experimental Therapeutics has the resources to evaluate drugs received.

POC: MAJ Steven Andersen, MD, MPH, WRAIR-ET, 301-427-5029

VENNERSTROM, J.L., ELLIS, W.Y., AGER, A.L., GERENA, L., and MILHOUS, W.K. 1992. bis-(4-aminoquinolines) 1. Antimalarials with potential against chloroquine resistance malaria. J. Med. Chem. (accepted).

Information Paper

Dept. of Pharmacology
Div. of Experimental Therapeutics
WRAIR

21 May 1992

SUBJECT: WR 238605 Development

OBJECTIVE: To design, implement, monitor, and evaluate the preclinical and clinical studies necessary for the development of a new antimalarial to replace primaquine.

STRATEGY:

I. Pre-Clinical
- A. Early Development to support IND
  - 1. Chemistry, Manufacturing and Control
    - a. Synthesis of bulk drug/radiolabeled drug
    - b. Characterize physical-chemical properties
    - c. Assay bulk drug for purity
    - d. Determine stability of bulk drug
    - e. Formulation of capsules and matching placebos
    - f. Formulation assay
  - 2. Pharmacology and Toxicology
    - a. Develop analytical methods for determination of drug concentration in various body fluids
    - b. Characterize the pharmacokinetics in several animal species
    - c. Characterize the activity in standard malaria test systems
    - d. Ames mutagenicity testing
    - e. Acute (LD_{50}) toxicity testing in various animal species
    - f. Subchronic (28 day) toxicity testing in rats/dogs
    - g. Pharmacodynamics/misc. toxicity
      - (1) Cardiovascular, Pulmonary, etc.
      - (2) Methemoglobin formation
- B. Advanced Development
  - 1. Tissue distribution studies in rats/monkeys ('91 - '92)
  - 2. 90-day toxicity study in rats/dogs with reversibility (Jul 92 - Jul 93)
  - 3. 1 year oral toxicity study in dogs ('94 - '95)
  - 4. 2 year oral toxicity study in rats ('94 - '96)
  - 5. Reproductive study in rats ('94 - '95)
  - 6. Teratogenicity study in rabbits ('94 - '95)
  - 7. Reformulation (?)

II. Clinical Pharmacology

A. Phase I

1. Single-dose safety and tolerance study (Jun - Sep 92)
2. Single-dose pharmacokinetic study ('92 - '93)
3. Multiple-dose safety, tolerance and pharmacokinetic study ('93 - '94)
4. Drug interaction studies (?)

B.Phase II

1. Causal prophylactic studies ('93)
2. Radical curative studies ('93 - '94)

CURRENT STATUS:

All pre-clinical studies necessary to support an IND have been completed (item I.A.). Of note is the long elimination half-life relative to primaquine. WR 238605 has been shown to be 7-10 × more active than primaquine, and significantly less toxic, in various *in vitro* and *in vivo* test systems. This greater therapeutic index, combined with the long half-life, may permit administration of a safer drug less frequently and for a shorter duration.

The IND has been submitted to the FDA. The Phase I Safety and Tolerance study protocol has been approved by the IRB, and the first human study should begin next month.

TECHNOLOGICAL BARRIERS:

None. However, insufficient resources exist to carry out planned toxicology studies in a timely manner.

PLANS:

Recommend awarding of an additional toxicology contract.

RESOURCES:

I. Funds to directly and indirectly support clinical studies

A. need for new formulation (formulation contract)
B. need for continued assay of drug in biologic fluids (analytical contract)
C. need for additional toxicology studies (toxicology contracts)
D. need for human kinetic studies (in house, contract)
E. need for human efficacy studies (in house, overseas)

MAJ Ralf Brueckner, MC, USA
301-427-5260

INFORMATION PAPER

Division of Experimental Therapeutics
Walter Reed Army Institute of Research

21 May 92

SUBJECT: Summary of Preclinical Efficacy of WR238605

BACKGROUND: Currently, only primaquine is clinically effective against persistent tissue stages of *Plasmodium vivax* or *P. ovale* in man. However primaquine has an extremely low therapeutic index. The most serious toxicity is hemolysis which occurs in individuals who are genetically deficient in glucose-6-phosphate dehydrogenase. Other adverse effects include: abdominal pain, cramps, anorexia, nausea, vomiting, leukocytosis, leukopenia, and methemoglobinemia. Headaches, interference with visual accommodation, pruritus, hypertension and arrhythmias have also occasionally been reported with primaquine therapy. The usual adult dosage of primaquine is 15 mg (26.3 mg of primaquine phosphate) daily for 14 days. Alternatively, adults may receive 45 mg primaquine (79 mg primaquine phosphate) once weekly for 8 weeks. Primaquine is well absorbed from the gastrointestinal tract with peak plasma levels attained within 1 to 6 hours. The drug is extensively and rapidly metabolized with a plasma half-life of 3.7 to 9.6 hours with less than 1% of the dose excreted as unchanged drug in urine.

OBJECTIVE: The difficulties with primaquine therapy summarized above in combination with its long and complicated dosing regimens limit its general usefulness as a radical curative and causal prophylactic antimalarial. Thus, the overall objective of this drug development effort is to produce a new, more effective, safer, radical curative and causal prophylactic drug to replace primaquine.

PROGRESS: WR238605 succinate is the leading drug candidate to replace primaquine. It is a new synthetic analogue of primaquine having an additional methoxy group at the 2 position, a methyl group at the 4 position, and a 3-trifluoromethylphenoxy substitution at the 5 position of the quinoline ring. WR238605 succinate is seven and ten timesmore potent than primaquine in the rhesus monkey as a radical curative and causal prophylactic respectively, and also has modest blood schizonticidal properties. In contrast to primaquine, the drug is effective as a single dose in the rhesus monkey radical curative model. Furthermore, WR 238605 succinate has a much longer half-life than primaquine in the rat (60 hours vs. 2 hours) , dog (54-165 hours vs unknown), and monkey (55 hours vs 1.6 hours). Recently, Bartlett et. al., 1991 and Queener et al., 1992, demonstrated that WR238605 was also effective for the prophylaxis and treatment of Pneumocystis pneumonia in rodent models.

FUTURE PLANS: The improved efficacy of WR238605, combined with its reduced toxicity, good oral bioavailability & longer half life make this drug an excellent potential candidate for causal prophylaxis and radical cures of drug resistant falciparum or vivax malarias in man. It's greatly improved therapeutic index compared to primaquine, suggest that it is likely to be a safe drug in clinical use and that it may be possible to design substantially shorter drug regimens for radical cures than are currently required with primaquine.

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POC: LTC Wil Milhous, PhD, WRAIR-ET 301-427-5029

BLOOD SCHIZONTOCIDAL TESTING
In the *Aotus* Monkey Model

WR238605 was effective in curing established trophozoite-induced infections of drug-resistant strains of *Plasmodium falciparum* in Panamanian *Aotus trivirgatus* monkey. Three-day oral regimens totalling 12 mg/kg parasitemia of primary infections of the drug-sensitive Uganda Palo Alto strain and cures were achieved at 48 mg/kg (total dose). Clearance of parasitemia of primary infections of the chloroquine- and pyrimethamine-resistant Vietnam Smith strain was not achieved by 12 mg/kg (total dose), although there were cures upon re-treatment of recrudescent infections at this dosage. WR238605 was effective against established blood-induced infections of *Plasmodium vivax* (Chesson strain) in Panamanian *Aotus trivirgatus* monkeys. Clearance of parasitemia was obtained at 3 mg/kg total dose and cures were achieved at both 12 and 48 mg/kg total dose administered in a 3-day oral regimen.

AGAINST VIVAX MALARIA

TOTAL DOSE	RESPONSE OF CHESSON STRAIN	
3 mg/kg	Clearance	2 of 2
12 mg/kg	Clearance	2 of 2
	Curative	2 of 2
48 mg/kg	Curative	2 of 2

AGAINST FALCIPARUM MALARIA

	RESPONSE OF STRAINS			
TOTAL DOSE	UGANDA STRAIN		SMITH STRAIN	
12 mg/kg	Clearance	2 of 2	No Effect	2 of 2
48 mg/k	Clearance	4 of 4	Clearance	6 of 6
	Curative	3 of 4	Curative	5 of 6
96 mg/kg	Curative	2 of 2	Curative	2 of 2

RADICAL CURATIVE PROPERTIES

WR238605 was effective in radical cure of established sporozoite-induced *P. cynomolgi* (Bastianelli) malaria in rhesus monkeys . Tissue schizonticidal cures were achieved at dosages of 0.1, 0.316 and 1.0 mg/kg/day (base) for seven consecutive days by oral administration in combination with a completely suppressive blood schizonticidal regimen of chloroquine. WR238605 was an effective agent against the persistent tissue stages of this parasite with a calculated CD_{50} (curative dose in 50% of animals) of 0.172 mg/kg/day (base) X seven days. Thus, WR 238605 was 7.4 times as active as primaquine as a tissue schizonticide.

Seven Daily Doses (plus Chloroquine)	Cures
1.0	2 of 2
0.31	8 of 8
0.178	0 of 4
0.100	2 of 6
0.316	0 of 2

Calculated CD_{50} = 0.172 mg/kg/day x 3 day

Molar Primaquine Index = 7.4

WR238605 was effective in radical cure of established sporozoite-induced *P. cynomolgi* (Bastianelli) malaria in rhesus monkeys when administered as a single dose. Tissue schizonticidal cures were achieved at single dose regimens of 1.75 mg base/kg (3.8 µmoles/kg) when administered with a completely suppressive dosage regimen of chloroquine. In contrast, single dose cures were consistently achieved with primaquine at 7.0 mg base/kg (26.6 µmoles/kg) when chloroquine was co-administered. Neither WR238605 succinate nor primaquine were effective as radical curatives in a single dose regimen without chloroquine co-administration.

CAUSAL PROPHYLACTIC PROPERTIES
In the Rhesus Monkey Model

WR238605 was also demonstrated to be effective as a causal prophylactic agent against pre-erythrocytic tissue stages of sporozoite-induced *P. cynomolgi* (Bastianelli) malaria in rhesus monkeys. Animals were challenged with sporozoites on day zero and drug administered on day -1, 0 and +1. Patency was successfully prevented at daily oral doses of 0.1, 0.316, 1.0 and 1.78 mg/kg/day (base). The causal prophylactic ED_{50} was calculated at 0.124 mg/kg/day (base) x three days. WR 238605 was, thus, 10.5 times as effective as primaquine against pre-erythrocytic stages of *P. cynomolgi*.

Three Day Regimen

Daily Doses	Patency Prevented
1.78	2 of 2
1.0	2 of 2
0.312	7 of 7
0.100	1 of 6
0.0316	0 of 4

Calculated CD_{50} by Linear Regression = 0.125 mg/kg/day x 3 days

Molar Primaquine Index = 10.5

Single Dose Regimen

Since WR238605 has been found to be active at 0.312 mg/kg in three day multiple doses, preliminary experiments wre conducted using 0.95 mg/kg (three times this dose) administered on days -2, -1 or 0 of sporozoite challenge. Patency was successfully prevented in only one animal. This single dose regimen was increased to 2.84 mg/kg and was protective on day 0. Increasing the dose to 5.68 mg/kg resulted in protection from day -4 in one of three monkeys and day -3 in three of three monkeys. Primaquine control doses (three times the effective dose when administered over three days) was effective only when administered on day 0. A single primaquine dose of 16.02 mg/kg was not effective on day -1.

CAUSAL PROPHYLAXIS
In the Rhesus Monkey Model
Single Dose Regimen

	Day Single Dose Administered				
DOSE (MG/KG)	-4	-3	-2	-1	0
	Numbers of Monkeys Protected				
WR238605					
0.95			0 of 2	0 of 2	1 of 2
1.42			0 of 3	0 of 2	2 of 2
2.84	0 of 3	2 of 6	2 of 2	3 of 3	2 of 2
5.68	1 of 3	3 of 3			
Primaquine Controls					
5.34				0 of 2	2 of 2
16.02				0 of 2	0 of 2

CAUSAL PROPHYLACTIC PROPERTIES
In Rodent Models

A single subcutaneous dose of WR238605 in peanut oil vehicle effected cures of mice infected three days previously by blood inoculation of *P. berghei* at doses of 40 to 320 mg/kg and 20 to 160 mg/kg . Early (before day 5) mortality attributable to acute toxicity was observed at 640 mg/kg and there was indication of delayed toxicity at 320 and 640 mg/kg.

In tests against sporozoite-induced rodent malaria, the compound exhibited prophylactic activity. A single dose of WR238605 administered either orally or subcutaneously to mice inoculated with sporozoites of *P. berghei yoelii* prevented infection at doses as low as 10 mg/kg by either route of administration. Mortality possibly attributable to drug toxicity was observed after subcutaneous administration at 40 and 160 mg/kg by either route of administration. A single dose administered subcutaneously to mice challenged with sporozoites of *P. y. nigeriensis* was active prophylactically. The compound was slightly active at 3 mg/kg, active at 10 mg/kg and fully active at 30 mg/kg, indicating that the prophylactic activity might have been at least partially due to persistent blood schizonticidal activity rather than to causal prophylactic activity alone.

In tests for repository prophylactic activity, WR238605 was administered both orally and subcutaneously at 80 mg/kg (160 mg/kg total dose) to mice which were challenged three days later by injection of sporozoites of *P. y. yoelii* . No parasitemia was observed in treated animals through 17 days of observation. In further testing, WR 238605 was administered as above and mice were challenged 7, 14 or 21 days later. The compound exhibited little or no protective activity and there were deaths attributable to toxicity. Thus, WR238605 appears to have repository activity for at least three but less than seven days in this system.

IN VITRO BLOOD SCHIZONTICIDAL TESTING

Using a semi-automated micro-dilution technique,the intrinsic blood schizonticidal effectiveness of WR238605 was evaluated against standard cloned isolates of *Plasmodium falciparum* from West Africa, Brazil and Indochina. Uptake of ^{3}H-hypoxanthine was used as an index of parasite growth over a 66h incubation period. Concentration response curves were analyzed by nonlinear regression and fifty percent inhibitory concentrations calculated. On an equimolar basis, WR238605 was 25-60 fold more active than primaquine depending on the strain tested. WR238605 exhibits no obvious patterns of cross resistance to standard or developmental antimalarial drugs.

Fifty Percent Inhibitory Concentrations of WR238605 and other Antimalarial Drugs Against Falciparum Malaria Parasites

Antimalarial DRUG	Indochina W2 Clone	Indochina W2MEF	African D6 Clone	Thailand KAW	Brazil ITG276	African 3D7
WR238605	0239.6	0028.2	0140.6	0135.8	0135.7	0065.5
Primaquine	6258.3	0704.4	5868.0	4408.5	7802.2	4206.6
Calculated Primaquine Indices	**22.2**	**25.1**	**42.0**	**32.6**	**57.8**	**64.2**
Quinine	0058.5	0048.2	0031.1	0067.8	0168.1	0110.5
Chloroquine	0116.0	022.79	0007.4	0023.7	0040.7	0011.6
Mefloquine	0002.3	0016.7	0015.3	0027.1	0007.9	0025.5
Artemisinin	0002.8	0002.5	0005.6	0006.7	0003.6	0006.8

CORE CONTRACTS/SPLIT FUNDING

1.	CONTRACT DAMD17-91-C-1135 Dr. Peter Lim SRI International	Chemical Analysis
2.	CONTRACT DAMD17-92-C-2044 Mrs. Tommie Curtis Herner & Co.	Chemical Repository
3.	CONTRACT DAMD17-89-C-9058 Dr. Jaroslav F. Novotny Starks Associates, Inc.	Preparation Laboratory
4.	CONTRACT DAMD17-89-C-9057 Dr. Peter Blumbergs Ash Stevens, Inc.	Preparation Laboratory
5.	CONTRACT DAMD17-89-C-9064 Dr. Gregory Hatfield Pharm-Eco Laboratories	Preparation Laboratory
6.	CONTRACT DAMD17-89-C-9062 Dr. John A. Kepler Research Triangle Institute	Radiolabeled Synthesis
7.	CONTRACT DAMD17-92-C-2035 Dr. Douglas Flanagan University of Iowa	Formulation
8.	CONTRACT DAMD17-92-C-2028 Dr. Emil T. Lin Univ. of California at San Francisco	Methods Development in in Body Fluids
9.	CONTRACT DAMD17-87-C-7006 Dr. David R. Hawkins Huntingdon Research Centre	Pharmokinetic/Metabolism
10.	CONTRACT DAMD17-86-C-6177 Dr. Marvin M. Goldman University of California at Davis	Pharmokinetic/Metabolism

ANTI-PARASITIC DRUG DEVELOPMENT/RAD I FUNDED

A	DAMD17-85-C-5172 Chemotherapy of Rodent Malaria Professor Wallace Peters London School of Hygiene and Tropical Medicine London, England	Primary and Secondary Screening of Drug & Drug Combinations Against Rodent Malaria
B	DAMD17-91-C-1010 Evaluation of Chemotherapeutics Agents Against Malaria Dr. Arba L. Ager, Jr. Center for Tropical Parasitic Diseaes 12500 S.W. 152nd St. Miami, Florida 33177	Primary and Secondary Screening of Drugs Against Strains of Rodent Malria
C	DAMD17-91-C-1072 Drug Evaluation in the *Plasmodium falciparum Aotus* Model Dr. Richard Rosson Gorgas Lab c/o ProMed Trading Panama City, Panama	Advanced Testing of Human Falciaprum Malaria in Primates
D	DAMD17-93-Z-3XXX Evaluation of New Antimalarials Dr. B. N. Dhawan Central Drug Research Institute Lucknow, India	Advanced Testing of Relapsing (vivax-like) Malaria in Primate
E	DAMD17-90-C-0131 Study of Compounds for Activity Against Leishmaniasis Dr. William L. Hanson University of Georgia Athens, Georgia	Screening of Potential Anti-Leishmania Drugs in Rodent Models
F	DAMD17-87-C-7146 Biochemistry and Chemotherapy of Leishmaniasis Dr. Linda Nolen School of Public Health University of Massachusetts Amherst, MA 01003	Basic Research in Parasite Biochemisty to Identify New Drug Targets and Determine Mechanisms of Action
G	DAMD17-87-C-1012 Mechanisms of Drug Resistance in *Plasmodium falicparum* Dr. Dyann Wirth School of Public Health Harvard University Cambridge, MA 02138	Molecular Biology & Biochemisty of Antimalarial Drug Resistance

H DAMD17-91-C-1015
Methods for Evaluating Drug Resistance in *Plasmodium falciparum*
Dr. Michael T. Makler
Flow INC 6127 S.W. Corbett
Portland, Oregon

Development of Rapid Field Method to to Assess Drug Resistance

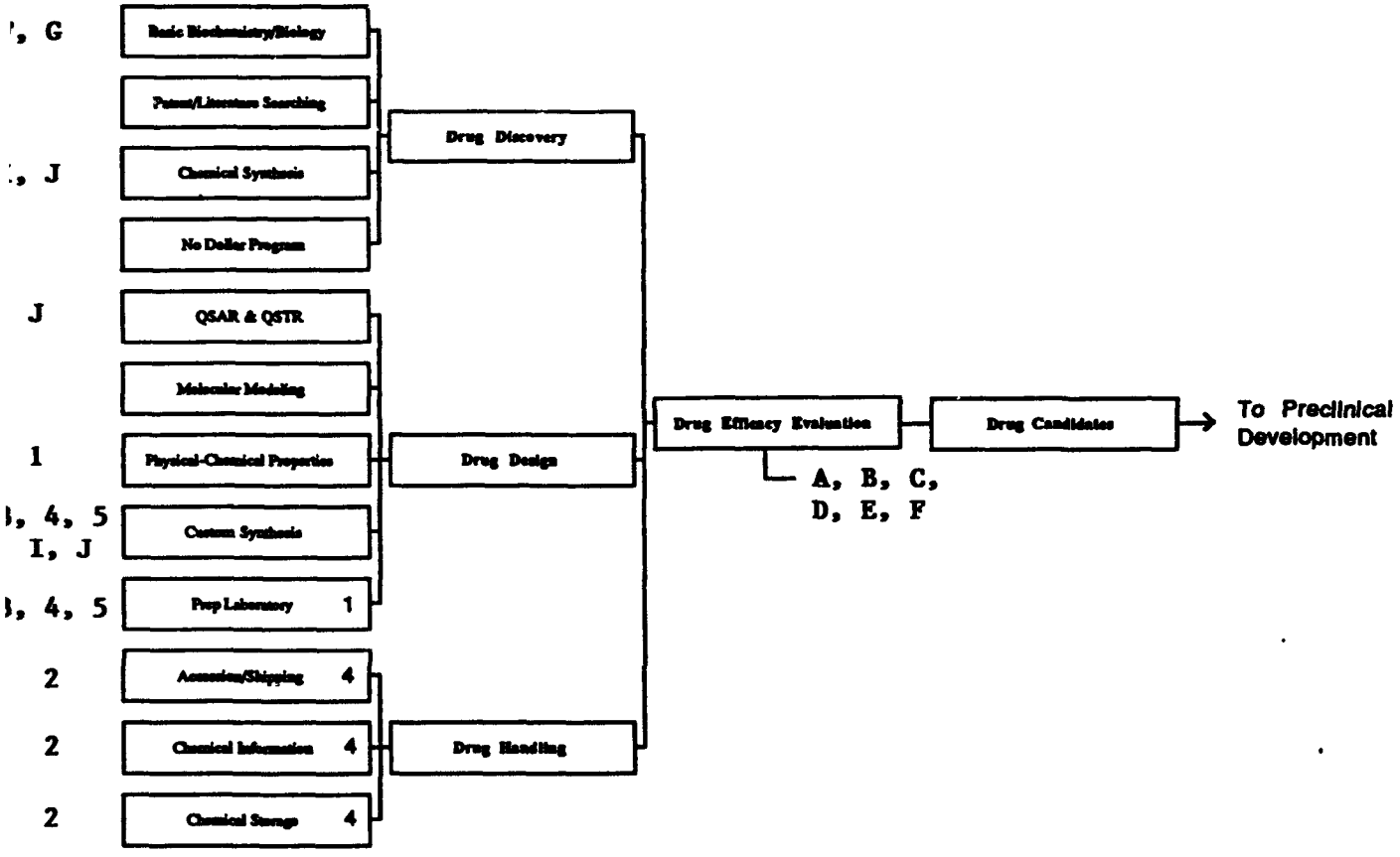
I DAMD17-93-C-Pending
Dr. Gary Posner
Johns Hopkins
Baltimore MD

Synthesis of Synthetic Peroxides

J DAMD17-91-C-1099
Dr. Mitchell Avery
Synthesis of Artemisinin Analogs
University of North Dakota

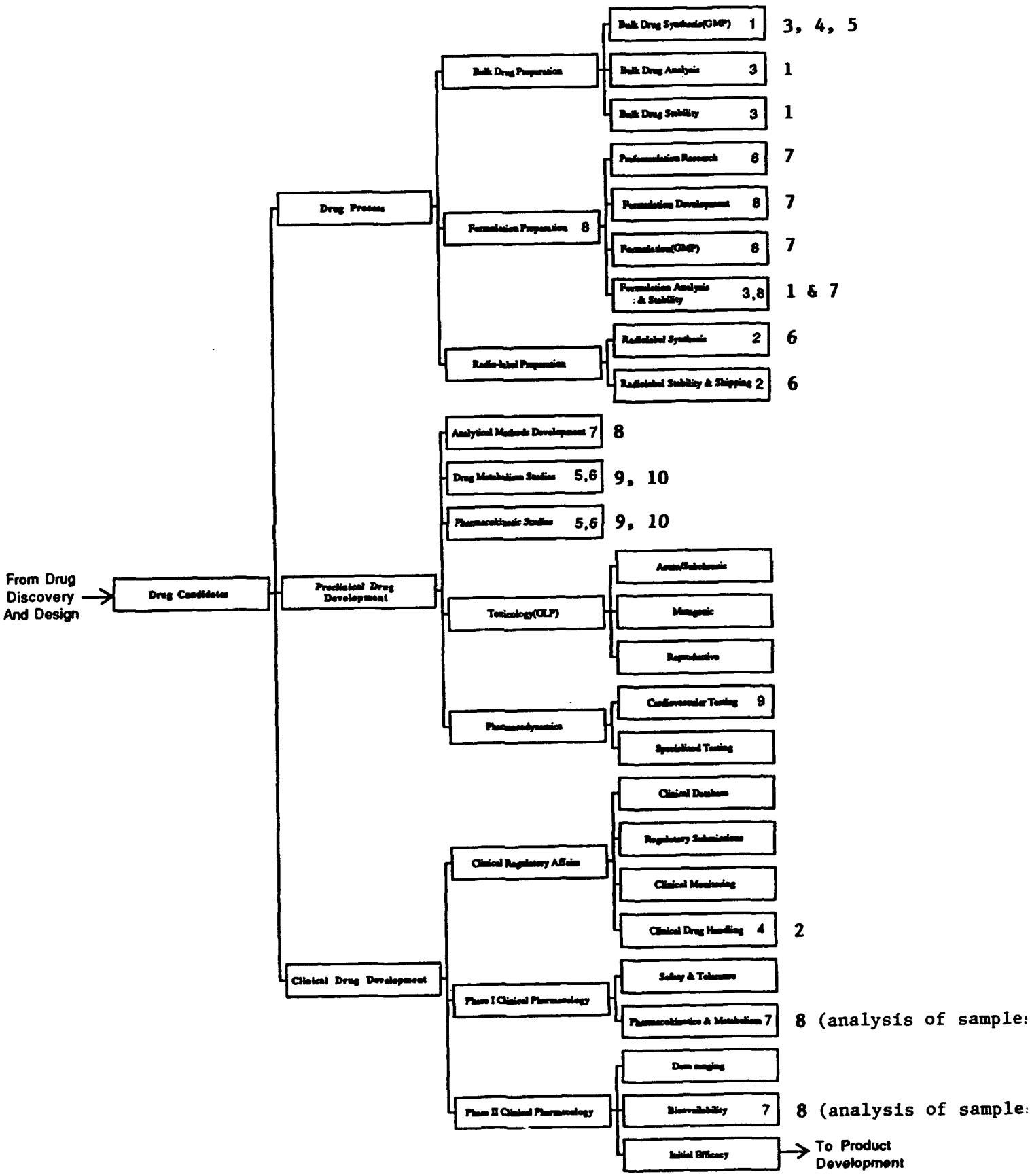
Sythesis Contract

WRAIR Division of Experimental Therapeutics
1 - Drug Discovery/Design



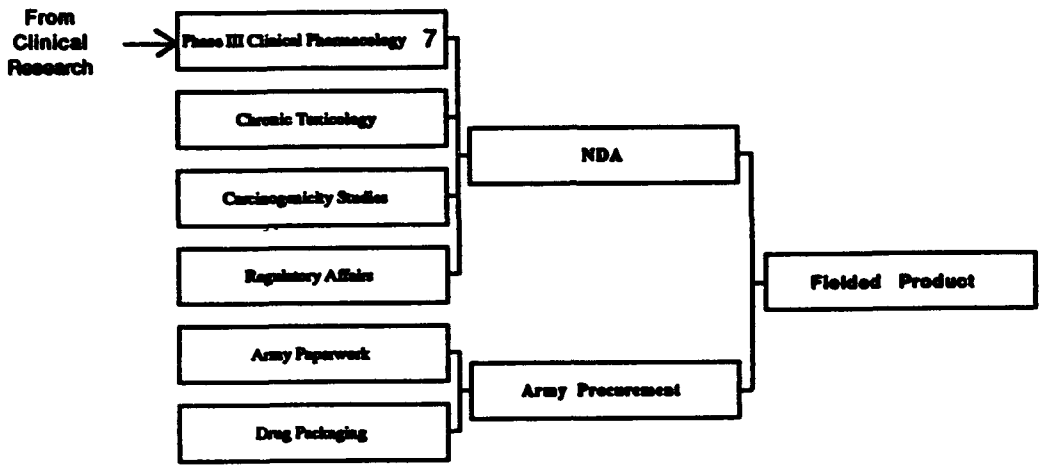
WRAIR Division of Experimental Therapeutics

2 - Preclinical/Clinical Drug Development



WRAIR Division of Experimental Therapeutics

3 - Product Development



1992 DOD Malaria Research Program Review

Executive Session

COL Bancroft began the discussion by reviewing some differences between the malaria vaccine and drug development programs. The ten-year-old malaria vaccine research program is a joint-service program conducted almost exclusively in-house. While it has not yet produced a final product, its goal is to produce a complex multistage polyvalent vaccine, the first of its kind. The twenty-five-year-old malaria drug development program is conducted almost exclusively by the Army and is very dependent on extramural contracts. It has produced several products and many more are on track. The drug program is more dependent on testing in animals, while the vaccine program is more dependent upon testing in humans.

Funding

COL Bancroft reviewed the Infectious Disease budgets for fiscal years (FYs) 1991-1995 and the breakdown by funding lines (6.1 to 6.4) (Figure 1). He noted that the total Infectious Disease budgets for 1991-1992 included supplements of $12.5 and $8.7 million respectively; it is not known whether Congress will provide a supplement for FY 93. In general, it is expected that there will be less money to support the malaria research program in the future, especially for technology-based research.

The malaria budget is $9.7 million or 19 percent of the $45 million FY 92 Infectious Disease budget, with funding split almost equally between research on drugs and vaccines (Figure 2). COL Bancroft said that malaria research does not compete for funds with either research on human immunodeficiency virus (HIV) ($50 million in 1992) or for Biological Defense ($60 million in 1992). On the other hand, all other Infectious Disease programs do compete with malaria research for funds. Although the HIV and Biological Defense programs have supporters in Congress, the Infectious Disease program has congressional critics who think that the technology-based work could be done at the National Institutes of Health (NIH).

The distribution of research funds for malaria vaccine and drug development are shown in Figures 3 and 4. These funds support OCONUS as well as CONUS research.

There was discussion of the misunderstanding of how overhead costs at the Walter Reed Army Institute of Research (WRAIR) are charged to the Infectious Disease 6.2 fund line. COL Bancroft explained that the U.S. Army Medical Research and Development Command (USAMRDC) pays administrative costs of each Army lab from a designated 6.2 line which has been adjusted to cover these expenses. The WRAIR uses the infectious disease line, and the 6.2 funds have been programmed for this purpose. The Navy overhead costs are paid from the funds provided by USAMRDC. Dr. Sell suggested there should be better definition of direct and indirect costs.

COL Bancroft said that extramural programs for malaria research have declined. From 1991-1993 the number of contracts will have gone from 5 to 1 in the malaria vaccine program and from 25 to 11 in the drug program. These cuts will result in far fewer contacts with academia and the research community. Prior to 1992, the CORE Drug Development contracts were funded by three programs—Infectious Disease, Biological Defense, and Chemical Defense. Since then, Biological

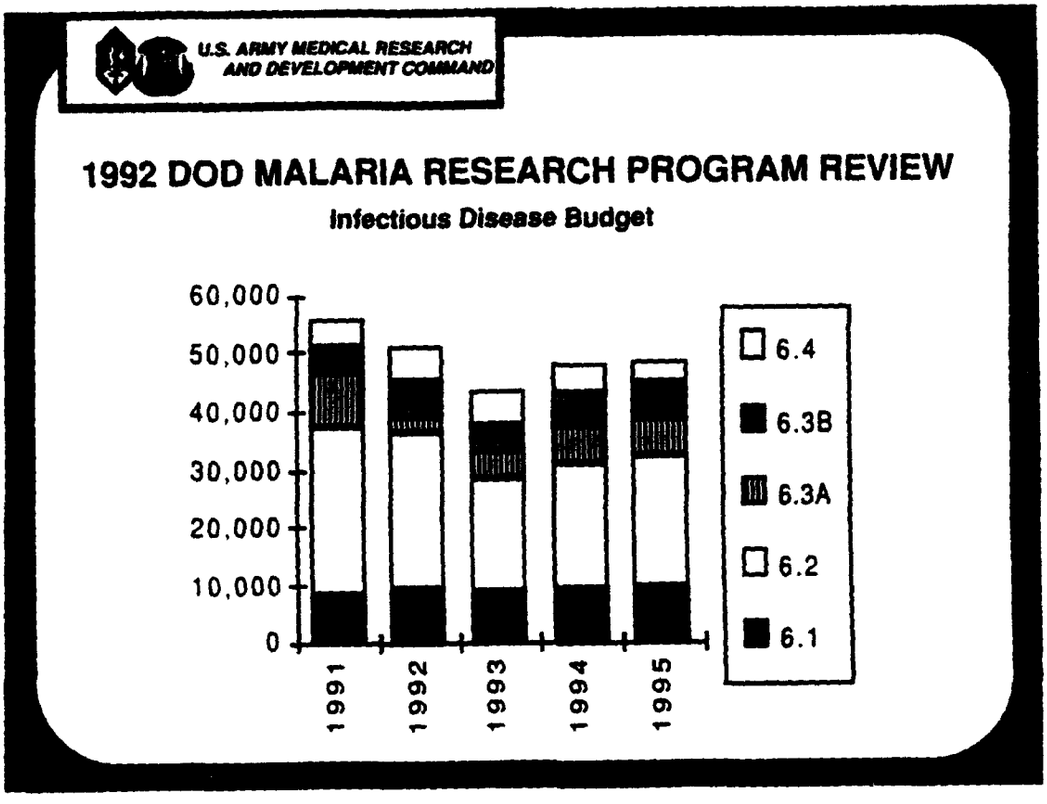


Figure 1

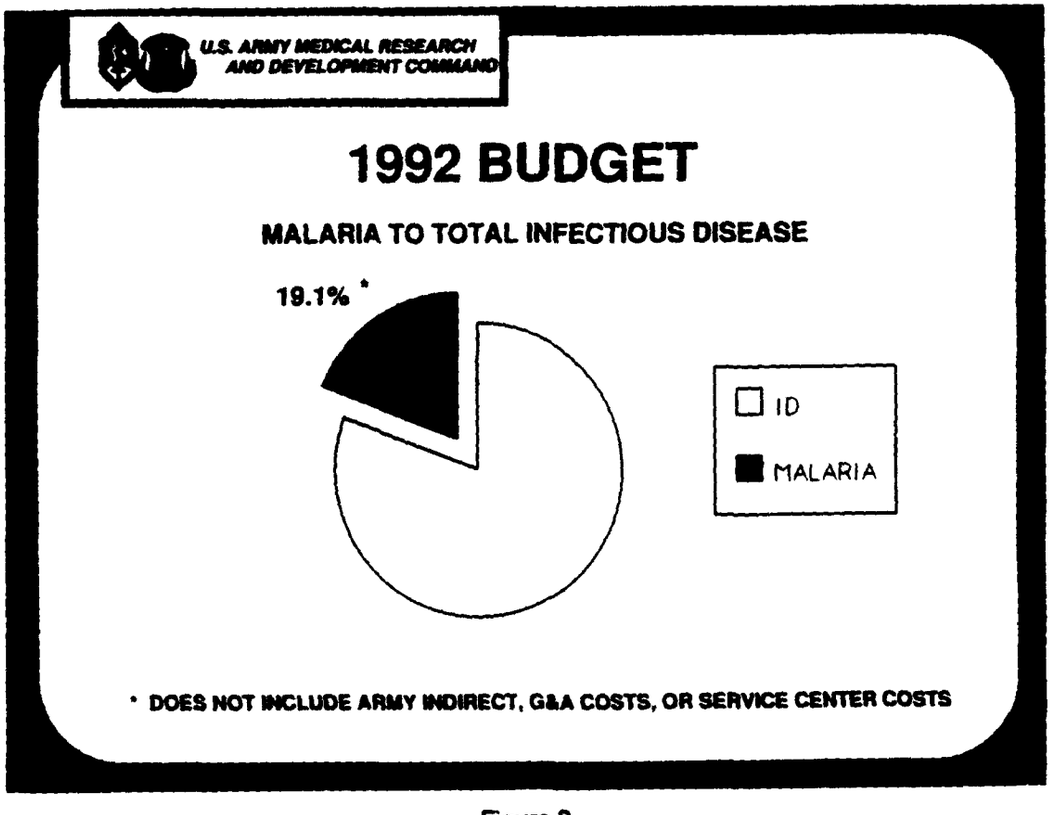


Figure 2

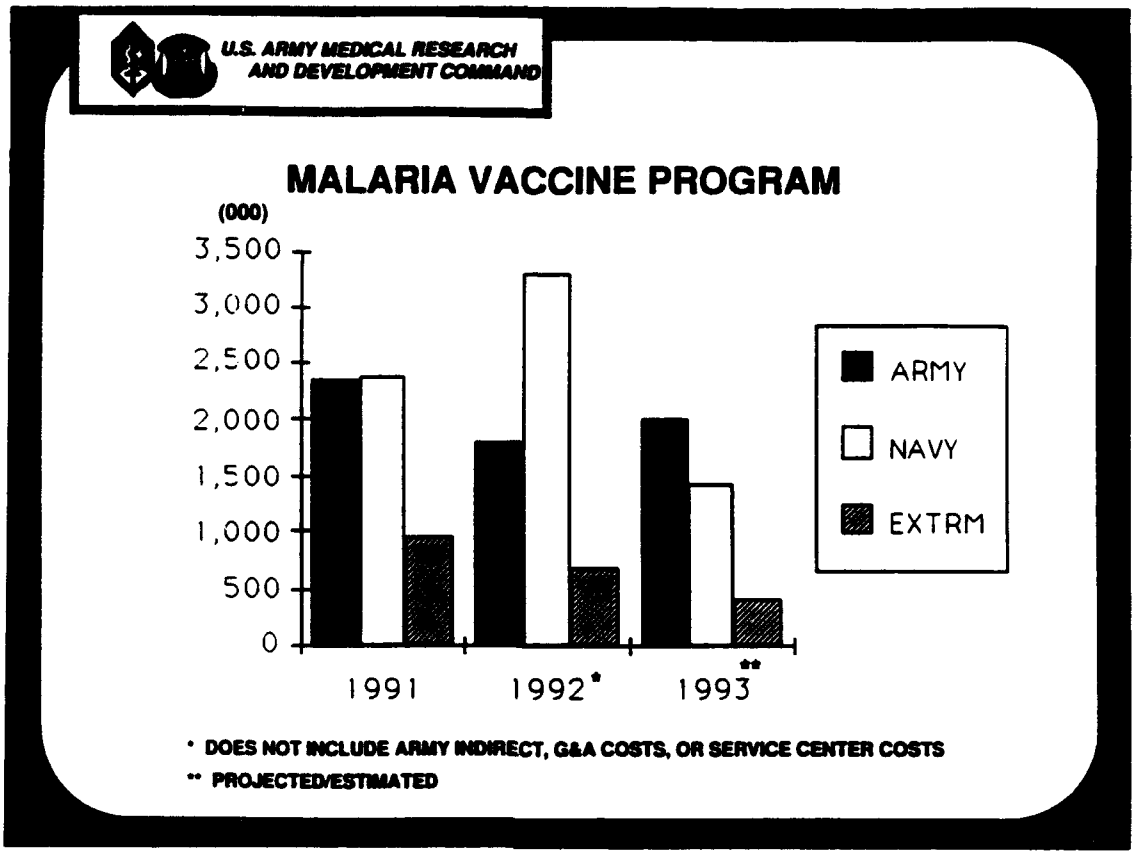


Figure 3

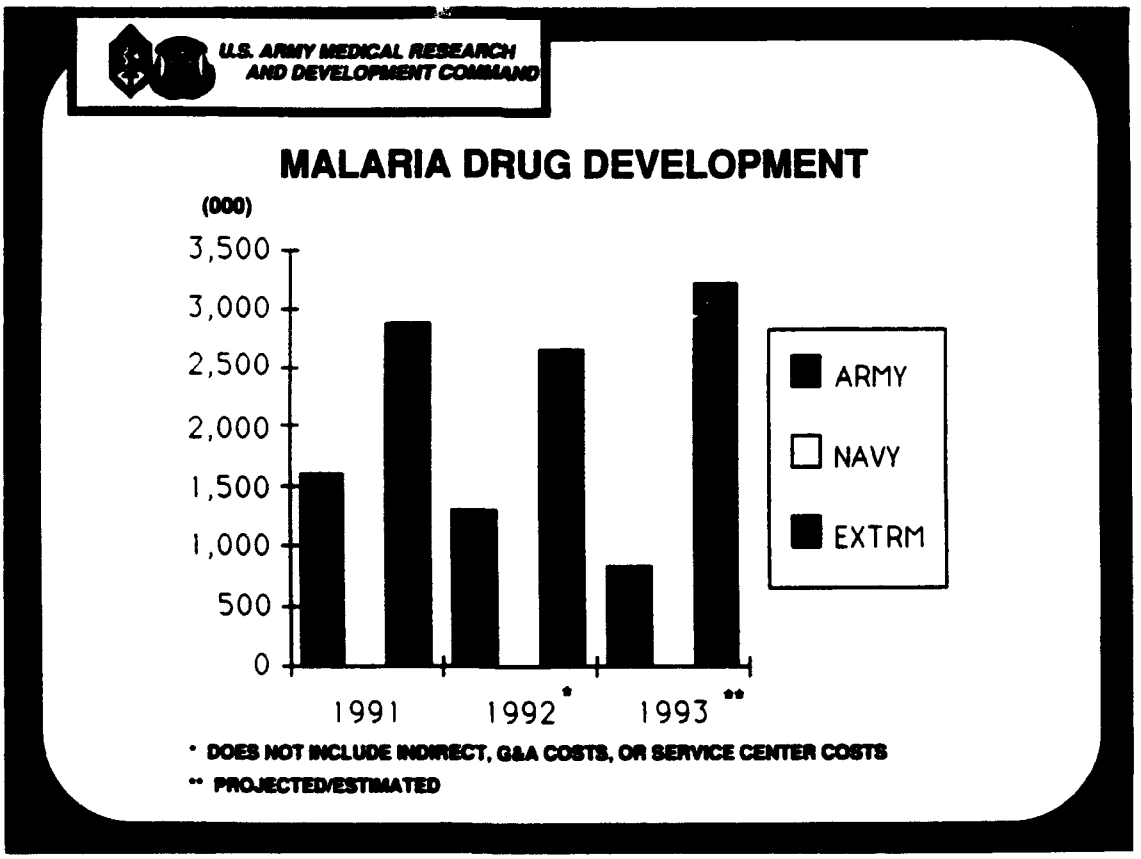


Figure 4

Defense withdrew support, and 60 percent of the funding was reassigned to Infectious Disease (in 1993 this will be 85 percent). As a result of these changes, the Infectious Disease Program will be required to pay substantially more for extramural antimalarial drug development even though there are fewer contracts.

General Discussion

CAPT Larry Laughton questioned the accuracy of the Navy malaria vaccine expenditure designated as $3.5 million in FY 1992. COL Bancroft said the slide was based on the best available data from the Navy and the Army comptroller. He explained these were the funds transferred to the Navy for malaria vaccine related work. In the past, funds were designated only by funding line, not by particular projects, and so expenditures were difficult to track. In the future, laboratories will have more explicit guidelines on how to use their funds due to the DOD mandate to budget by objective.

There was further discussion about the need to clarify the allocation of funds in the different funding lines. COL Jerald Sadoff said that funds in the 6.3b and 6.4 lines are not being spent appropriately, and there should be more money in the 6.1 line. Other questions were raised about the distribution of funds to overseas laboratories and the appropriate role of the Laboratory Commander.

COL George Lewis said the Pentagon is concerned about a lack of prioritization in the programs. CDR Richard Oberst stressed the need for documenting the importance of these programs to Congress.

Program Coordination

A lengthy discussion ensued about the need for greater coordination between the Army and Navy malaria programs. Participants agreed that there must be improved interaction between the two services in order to make the best use of available funds in an era of decreasing resources. Both Army and Navy representatives expressed their belief that a more integrated program would be beneficial.

CDR(P) Stephen L. Hoffman noted that the Malaria Vaccine Steering Committee, which advises and coordinates joint service activities of the malaria vaccine effort, has always had a chairman from the Army, and he suggested that the chairmanship alternate between the Army and Navy. LTC W. Ripley Ballou is the current chairman of the Steering Committee. The Committee meets every four to six weeks to provide status reports on projects, but it rarely addresses basic research and has no authority over budgets.

COL Sadoff said that coordination regarding the diarrhea research program is not a problem because the Army and Navy have clearly separate roles, and he suggested that there be a similar structure for the malaria program under the direction of one leader. He said that the DOD cannot afford to waste its limited resources, which are trivial compared with industry. COL Sadoff also stressed the need for open and immediate sharing of information between the Army or Navy.

Recommendations from Advisors

All three reviewers were impressed by the high quality of research and the progress made by the internationally respected DOD malaria research program. All agreed that the programs need more money to continue working at this high level.

Dr. Louis Miller made the following comments on the first day of the review. He was absent the second day due to a prior commitment.

- A system is needed to test vaccines in animals (perhaps monkeys) prior to field testing in humans.
- Cuts in the DOD malaria research budget could wipe out the chance of eradicating malaria.
- No other group is in the position to make this progress.

On the second day, Dr. Sell made the following points:

- The malaria vaccine and drug development programs need equal weight in the long-term and both must be supported.
- Coordination between the Army and Navy programs must be improved in order to make the best use of available money.
- An overall review of programs is needed to allow difficult decisions on research directions to be made. There should be a permanent advisory panel to review 6.1 and 6.2.
- Research personnel are the program's most important resource and must be nurtured.
- A commander with scientific expertise should provide leadership to the programs.
- Congress must be convinced of the importance of the programs.
- The DOD should not give up basic research.

Dr. Diggs made the following points:

- An interagency forum could look at ways to increase research and get more resources.
- Both Army and Navy vaccine programs may not be needed. One person should be in charge of the program.
- A zero-base budget should be used to determine what is absolutely critical and what is noncritical to the program. One way to determine the critical path to a product is to produce a five-year plan. Research in monkeys might be noncritical.
- The percent of the budget spent on basic research should be subjected to the scrutiny of peer review.
- RAD-1 input is needed to improve program coordination.

Feedback and Future Plans

COL Bancroft asked participants for reactions to the meeting format. Stating that a great deal of material had been covered in 1-1/2 days, he said that other topics could be covered in this concentrated fashion, and he asked the participants whether they would like to have similar meetings.

Several attendees felt the scientific review part of the meeting was unnecessary, and too detailed, while others thought the review was informative. Dr. Sell suggested that a member of the Federal Coordinating Council on Science, Engineering, and Technology (FCCSET) should attend these meetings. COL Schuster expressed the opinion that the meeting will have been beneficial as long as its outcome is to fight for more dollars, rather than to adopt a defensive stance to save losses.

COL Bancroft complimented the presenters for providing information that was responsive to the requests and thanked the participants for their input. He said the final report would include recommendations about the issues raised during the meeting.

Memorandum 5 June 1992

To: William Bancroft, M.D.
Colonel, Medical Corps
Director
Research Area I
US Army Research & Development Command
Fort Detrick
Frederick, MD 21702-5012

From: Carter Diggs, M.D., Ph.D.
11202 Landy Court
Kensington, MD 20895

Subject: Report of Review of USAMRDC Malaria R&D Program

Enclosed is a report of findings and recommendations made as a result of the two day review of the USAMRDC malaria Program on 20-21 May 1992 in which I participated. The report is in three parts. The first is a one page summary of the report. The second part addresses the specific concerns posed to the review team at the time of the meeting and the third lists specific recommendations for consideration.

Executive Summary

STATUS REPORT

•***Requirement for Research and Development:*** Malaria remains one of the most potentially devastating hazards to military operations. Currently available preventive measures are poorly effective. R & D towards improved measures (new drugs and vaccines) is essential.

•***Goal Orientation of Research***: The programs are sharply focussed on development of drugs and vaccines which will protect military personnel from malaria during operations in endemic areas. Scientists and support personnel are highly motivated and are moving forward with determination towards product development.

•***Scientific Quality***: In addition to being highly goal oriented, the programs are world class scientific endeavors. Program scientists are leaders in the academic community as well as contributors to product development.

•***Progress:*** New drugs for malaria are being produced as fast as possible within the constraints of the resources available. Progress towards vaccines has been remarkable, but much is left to be done before a vaccine can be fielded.

IMPEDIMENTS and PRINCIPAL RECOMMENDATIONS

•***Program management is too decentralized:*** Implementation of major program activities (for example vaccine trials) requires management across diverse organizational boundaries. **Resources should be allocated directly to managers with overall program responsibility and authority.**

• ***Resources are suboptimal***: The magnitude and importance of the problem is not accurately reflected by resources allocated. New drugs would be made available at a more rapid rate and effective vaccines would be developed sooner if the programs were better supported. **Funding and personnel authorizations at at least the FY90 level should be assured, even if decrements in other programs are necessary.**

Submitted by Carter L. Diggs, M.D., Ph.D., Consultant in Malaria Vaccine Development, 5 June 1992

Response to USAMRDC Concerns

The research

•*Goal orientation:* There is no question that both the drug and vaccine programs are sharply focussed on the goals: to develop malaria vaccines and drugs which will protect military personnel from malaria during operations in endemic areas. The secondary goal of development of chemotherapeutic agents is also being addressed effectively. Whereas some of the work was characterized as "basic research", the fundamental studies being done are, for the most part, in order to allow the next practical steps to be taken towards development of the products which are the objective of the program Activities range from the elucidation of immune mechanisms and identification of antigens and the mechanisms of drug action, through the design and construction of experimental drugs and vaccines, to clinical evaluation of the safety and efficacy of the agents. It is insight gained from the latter types of studies that identify the types of fundamental investigations needed to obtain answers which will expedite the next steps towards practical development.

•*Appropriateness of technology*: Leading edge technology is being used in all aspects of the program .

•*Progress:* As regards vaccines, in spite of the fact that ultimate success can only be accessed when an operationally effective vaccine is fielded, much progress has been attained in the last decade which was not only not anticipated but was not even imagined during the preceding decade. More importantly for the current review, progress in the last few years has been remarkable as well. The identification of cytotoxic targets on infected hepatocytes, the identification and characterization of new antigens, in both preerythrocytic and erythrocytic stages of *P. falciparum*, the clinical evaluation of an extraordinary number of experimental vaccines involving the introduction of novel adjuvants are all examples. As regards the drug program, progress is easily measurable in terms of the agents moving toward and being licensed over time. In

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addition, the new approaches enabled by the discovery of the reversal of drug resistance constitute remarkable advances. Formal comparison of progress with previously prepared plans was not part of the review exercise for either the drug or the vaccine program (see **recommendation 1**).

•*Unnecessary duplication*: Overall, there has been extraordinary success in coordinating work to avoid ill advised, unnecessary duplication of effort. The drug program is unique and tightly managed, and duplication within DoD is very unlikely. However, there is a danger that unnecessary duplication could take place in the vaccine program under the current management structure. This will be expanded on in the section on interaction, below.

•*Deficiencies:* Development efforts towards blood stage vaccines are moving much more slowly than those towards preerythrocytic vaccines. Whereas this prioritization is probably correct, the blood stage area is also important. Funding for fundamental research in this area appears to be quite deficient ($100K for fundamental research at WRAIR projected for FY93). Another major deficiency has been the necessity to depend on convincing industry of the market potential of vaccines in order to obtain GMP material. This situation will be vastly improved with completion of the new scale up facility at Forest Glen, but additional support will be required for its effective operation. Deficiencies related to interaction and resources are discussed further below.

Interaction

•*Within institutions:* There is a need for considering the DoD response to malaria in a holistic fashion to better assess the potential of each preventive modality and the time frame in which implementation is likely, and to better assess relative resource needs of various elements of the overall program. This need is addressed in **recommendation 2**.

Submitted by Carter L. Diggs, M.D., Ph.D., Consultant in Malaria Vaccine Development, 5 June 1992

Within WRAIR, there is excellent collaboration between the various departments of both the Division of Communicable Diseases and Immunology and the Division of Experimental Therapeutics due to the orientation of the scientists and the leadership of the Division Directors. NMRI also appears to have a very well integrated intramural program. However, collaboration between CONUS and OCONUS laboratories is sometimes less than optimal, perhaps in part because of the several management structures involved. Program based resource allocation would likely improve this situation (see **recommendation 3**).

•*Between labs:* In spite of intense efforts at coordination, the WRAIR and NMRI vaccine efforts constitute two programs. In view of the pooling of infectious disease funding in DoD and the virtually identity of the objectives of the two programs, it is difficult to justify their separate management. Although there is a risk that there would be some degradation of morale with consolidation of the currently very effective two teams, the benefits of such consolidation in terms of increase efficiency and effectiveness of the combined effort would seem to make increased efforts at consolidation of effort necessary (see **recommendation 4**).

•*Between federal agencies*: As already mentioned, the drug development program is unique, so there is little opportunity for interagency interaction. Malaria vaccine research and development in other federal agencies has a distinctly different objective than does the DoD effort. Whereas the DoD program has as its objective the development of vaccines which prevent malaria morbidity in military personnel, the NIAID program is devoted primarily to fundamental research and the A.I.D. program to development of vaccines designed to reduce mortality among residents of endemic areas. The latter objective differs from that of DoD in that the first goal is to prevent death from malaria without necessarily preventing morbidity, although the latter is obviously also desirable and is a longer term goal. Prevention of morbidity in military personnel is essential to prevent degradation of performance, and this requirement effectively

Submitted by Carter L. Diggs, M.D., Ph.D., Consultant in Malaria Vaccine Development, 5 June 1992

translates into the prevention of infection. The NIAID program is the major national effort which has as its objective the deepening of the knowledge base in malaria biology. This objective is an essential part of the national malaria program since it provides the bases for new approaches to the practical problems. For these and other reasons, autonomy of the three efforts needs to be maintained. Nevertheless, there is great potential for collaborative efforts among the three agencies and a need for continued efforts to enhance communication and coordination. The recent study by the Institute of Medicine (IOM) of the U.S. response to the malaria problem proposed that a national advisory body to help coordinate malaria efforts be formed. With this proposal as a point of departure, Louis Miller has taken the initiative to propose a malaria forum, although the precise activities to be initiated have not yet been formulated. The IOM is developing a proposal for this forum. The USAMRDC, which has been approached to help fund this effort should take a careful proactive role in the development of the initiative (see **recommendation 5**).

•*With academia and industry:* There is excellent interaction of DoD Malaria Vaccine Research and Development Program scientists with academia though scientific meetings as well as active scientific collaboration. In addition, Industry has been convinced to provide surprisingly large contributions to the effort. Whereas the former will continue, interest in malaria vaccine development in industry appears to be declining rapidly. Early enthusiasm that a marketable vaccine would be achievable in the short term has waned. The impact of this on resource requirements is discussed below.

Resources

•*Funding and staffing :* These two resource elements will be considered together. The algebraic curve which describes R & D progress as a function of resources is a sigmoid dose/response curve which becomes asymptotic at a level which can take on various values. Other conditions

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being equal, with a small increase in an originally undersupported effort, a proportionally even smaller incremental increase in progress is made. As resources increase, progress grows exponentially until current potential is realized at which time it levels off and becomes asymptotic. With the availability of new knowledge or new technology, the value at which the curve becomes asymptotic can become elevated. The curve for the DoD Malaria Vaccine Research & Development program is on the exponential part of the curve. Considerable effort is currently being spent on creative methods to leverage other activities to accomplish the work, but this falls short of providing what is needed. As indicated above, progress towards blood stage vaccines is considerably behind what would be possible with adequate resources. Although full exploration of the potential of preerythrocytic stage vaccines has correctly been given higher priority since prevention of infection is the desired quality in a vaccine for military personnel, blood stage vaccines also have considerable potential for the DoD objective, and slow progress in this area could slow the arrival of a useful product. Also, as indicated above, contributions to the vaccine effort by industry are decreasing, thus making greater demands on public sector spending to accomplish the work that must be done to develop vaccines.

The amount of additional funding and staffing which would optimize progress cannot be specified. Further, new management knowledge that comes with experience in managing a better supported operation can elevate the value of the asymptote. However, it is clear that resources should at least be maintained at the FY90 level of $4.8M (see **recommendation 6**).

The question of where resources will come from is critical. **Recommendation 7** addresses allocation of currently available funds.

All present at the review were generally already familiar with the impact malaria has had on military operations in the past, but it is not likely that those who most need to know, ie the decision makers who could provide additional resources for accelerated progress, have the same

Submitted by Carter L. Diggs, M.D., Ph.D., Consultant in Malaria Vaccine Development, 5 June 1992

understanding. The pending publication reviewing the history of the influence of malaria on past Naval and Marine operations will be a useful document in this regard. Information of this type pertaining to Army operations is available, but a review publication of this subject might also be in order. However, such documents will not suffice to inform decision makers. Transmission of the information to the appropriate individuals must be accomplished as a proactive collaborative effort involving USAMRDC and Army and Navy technical experts. (See **recommendation 8**).

Recommendations

1. Program reviews should place somewhat more emphasis on comparing prior program planning milestones with actual achievements. This "reality testing" exercise would help to make plans more realistic and thus provide for more effective management.

2. The Director, Infectious Diseases, USAMRDC (RAD I) should enlist a Liaison Officer for Malaria (LO-MAL) tasked with periodically briefing the Director, as well as others, on the current status of preventive measure against malaria and the research and development activities pertaining thereto. This task could be accomplished with approximately one tenth effort by the appropriate designee. The LO-MAL should keep well informed on current contingency planning by the Preventive Medicine Consultant, OTSG, and his counterpart in the other services as well as on the results of research and development efforts in the Services and worldwide including time lines for deployment of new preventive modalities. (S)he should also be cognizant of R&D needs and funding profiles to advise RAD-1 regarding resource allocation. (S)he should also maintain the capacity to identify experts to provide needed specific information in briefings and other venues.

3. The Director, WRAIR should appoint a LO-MAL tasked with

Submitted by Carter L. Diggs, M.D., Ph.D., Consultant in Malaria Vaccine Development, 5 June 1992

periodically briefing him as well as USAMRDC, on the status of malaria research and development efforts in the Institute. A similar time committee would be required as for the USAMRDC LO-MAL, less if both tasks were performed by the same individual. The WRAIR LO-MAL should be well informed of the efforts and knowledge base of the USAMRDC LO-MAL. (S)he should also be cognizant of R&D needs and funding profiles to advise RAD-1 regarding resource for malaria within WRAIR.

4. RAD-I, in consultation with the Director WRAIR and the Commanding Officer, NMRI, should provide all 6. 1, 6. 2, and 6. 3A malaria vaccine development funds to the Malaria Vaccine Development Steering Committee Chairman for his or her allocation, based on programmatic need, to the diverse elements of the Program at WRAIR, at NMRI, in OCONUS laboratories, and in extramural work sites. Accounting should be accomplished through the organizational element to which the Malaria Vaccine Development Steering Committee Chairman is assigned (ie Chief, Department of Immunology, WRAIR or Director, NMRI Malaria Vaccine Development Program, etc.).

5. USAMRDC should work closely with the Institute of Medicine in an attempt to develop a useful and cost effective forum for interagency discussion and coordination in the U. S. response to the malaria problem. Issues which need to be addressed include (1) the development of a coherent national policy and strategy regarding the malaria problem, (2) means for securing adequate resources for implementation of programs, and (3) analysis of specific agency contributions to generic problems to better match resource provision with mission breadth (for example, contributions of USAMRDC to development of drugs which are used globally).

6. In view of the importance of USAMRDC malaria programs, funding and staffing should be maintained at least at the FY90 level, even if less critical programs have to suffer decrements or be terminated.

Submitted by Carter L. Diggs, M.D., Ph.D., Consultant in Malaria Vaccine Development, 5 June 1992

7 USAMRDC should review its entire program to reassess resource (funds and personnel authorizations) allocations in view of a updated assessment of current potential hazards to military operations. Elements with negligent relevance should be terminated and essential elements provided resources in proportion to the importance of the hazard. Zero based funding should apply with only core support provided without specific program justification. In additional to salaries and housekeeping, core support should include a segment (10%?) of the budget which should be used to support investigator initiated research. Since relative ILIR, 6. 1, 6. 2, and 6. 3A allocations are often distorted in a manner unrelated to actual need, such small scale initiatives needs to be protected by inclusion in core funding of each investigators laboratory. Additional fundamental investigations, as well as more advanced developmental efforts, should be funded contingent on formal protocol approval after programmatic and scientific review by the appropriate steering committee chairman.

8. As new leaders assume positions of authority, knowledge held by those that they *replace must be reintroduced*. RAD-I should sustain a proactive ongoing effort to inform decision makers of the hazards of malaria (relative, of course, to other infectious disease hazards) to military operations and to secure additional funding for R&D activities.

Submitted by Carter L. Diggs, M.D., Ph.D., Consultant in Malaria Vaccine Development, 5 June 1992

June 9, 1992

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To: Col William Bancroft, US Army R & D Command
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Evaluation of the DOD malaria vaccine program

My evaluation was limited to the vaccine program because of a scheduling problem that precluded my participation in the review of chemotherapy. This was unfortunate, as the two programs go hand-in-hand to protect the soldiers from malaria during military operations. Today we have less to offer the soldiers than during the Second World War. During the Second World War, Atabrine became available, but despite its efficacy there was a real problem in the Pacific with malaria, probably because of not taking chemoprophylaxis regularly. Vietnam was the first war where the 4-aminoquinoline was not effective. Bill Tigertt, the director of WRAIR during the Vietnam War, went to the area for one year and developed regimens for evaluating treatment for drug-resistant malaria. Within a year there were greatly improved treatment regimens. Prophylaxis was less effective. The development of mefloquine began during the Second World War and was completed during the Vietnam War. It was highly effective against chloroquine-resistant parasites, but now in Southeast Asia it is no longer effective. High cost is limiting its use in Africa and protecting its efficacy. Halofantrine is becoming ineffective because of cross-resistance of mefloquine-resistant parasites. It is of the utmost importance to the military that vaccines be developed as rapidly as possible to fill the void in protective techniques for the soldiers and because vaccines do not depend on compliance of soldiers in the field.

At the same time, vaccines against malaria present a major scientific challenge. It is important when defending the program to keep in mind the difficulty because it would not be prudent to promise an effective vaccine in a given time frame, only the best shot possible with the technology available. A vaccine will be available because it is possible to immunize with irradiated sporozoites, but the exact time frame is unknown.

I am going to review the program in terms of quality of the program, structure of the program, and future needs.

A. Basic research: This has been outstanding, with multiple major discoveries in the past continuing into the present (leader in cytoadherence of infected red cells, the discovery of the sialic acid binding protein, and the relation between TRAP and SSP-2).

B. Development into trials: Here there has been great success at testing multiple constructs of the repeat of CSP. These include the original construct R32tet32, which has 32 repeats of NANP (two of a variant repeat) fused to the tetracycline-resistant-gene readout of frame. The individual with the highest titer was protected, and two with lower titers had delayed prepatent period. There was little boosting of antibody titer in the low number of volunteers with a rise to the same level after repeated immunization. There have been two changes in the intervening years: the carrier (fusion to NS-1 of influenza) and

the adjuvant. Potent adjuvants such as lipid A in liposomes and various carriers have been tested, giving high antibody titers, but these titers do not predict protection.

Live vectors such as vaccinia and Salmonella are soon to be tested.

The search for new targets such as SSP-2, which has been the focus of some studies, should lead to new constructs.

C. Approaches to vaccine development for the future:

1. Continue the program and hope that an effective vaccine will evolve from the partially effective vaccines. It is unclear why some people are protected and others are not. Unless it is possible to obtain additive effects of two targets, it may be difficult to arrive at the correct construct. Dr. Sadoff argued for more testing because of the low cost of field studies. I am not sure that the costs are quite what he says when one considers the cost of developing the constructs through phases 1 and 2 and the cost of field personnel. However, the program is developing much new information that could be most useful and may even lead to a vaccine. It is difficult to argue against the applied nature of the program until the constructs that are in the pipeline have been adequately tested. This takes advantage of the program in place and the large amount of effort to get to this point. I would not want to second-guess the program, which may be successful and bring us closer to the elusive vaccine.

2. I was concerned that the basic program at WRAIR was somewhat constricted because of resources and the demand of the applied program. I realize that the Naval program under Hoffman is supposed to carry on the basic work (and they are doing an outstanding job), but this is a big problem needing the best of both groups. Suppose the present approach needs some basic insight that is missing. Are you focusing on an applied program too early in the development process? For example, if there was an Aotus model for testing constructs, this would be most useful. I wondered if the carriers or adjuvants used on NS1 constructs would be better with R32tet32. There is the question of whether the folding of the repeats is critical to inducing the correct antibody to block invasion. Testing the fixed repeats (Saitterwait) may lead to protective antibodies. Unfortunately, the number of ideas tested have been limited in part because we do not know the reason for success or failure and in part because of the costs of tests. Would a model solve some of these problems?

D. Combined Army-Navy program: I feel that the DOD has gone a long way toward producing a coordinated program between the Army and the Navy. It had, and still has, challenges; but in a time of reduced money, is there any choice? One thought is that the program should no longer be called Army and Navy but should be only referred to as DOD. Furthermore, it may be helpful to have naval people assigned to WRAIR and Army personnel to NMRDC. I realize the potential problem of ratings when the individual is working under one supervisor and rated by another. Again, this should be seen as a challenge that must be met.

E. Overall evaluation: I think that the DOD should be most proud of a world-class program with world-class scientists. It is addressing the problem of developing a malaria vaccine that is most relevant to the military. It is attempting to find the correct balance between applied and basic research. I would tend to continue the basic program (as well as the applied program) until something looks more promising, but that will require extra resources that must be found in other programs. Clearly, malaria and diarrheal diseases are two areas where the military research groups are making major contributions and

deserve continued support. The challenge of developing and maintaining a unified program as the DOD program and not Army or Navy will be a continual challenge in the years ahead. With limited resources, this challenge must be met.

MEMORANDUM

TO: Col. William Bancroft
U.S. Army Research and Development Command

FROM: Kenneth W. Sell, M.D., Ph.D.
Professor and Chairman
Department of Pathology

DATE: May 28, 1992

SUBJECT: Evaluation of the DOD Malaria Vaccine Program

I. VACCINE DEVELOPMENT

1. CLEAR RESEARCH GOALS -

I was enthusiastic about the current accomplishments and quality of research now being undertaken in the development of malaria vaccines. The goals for attack of the malaria infection using vaccines targeted at individual stages of malaria infection seem very appropriate. In the area of sporozoite vaccine development, the exciting new findings regarding the role of CTL T8 lymphocyte-medicated immunity seems quite clear with dual target antigens now being studied (CSP and SSP-2). The goals presented are both short term and long term. On the one hand, development of short-term passive immunity would be adequate for temporary protection of DOD personnel not unlike that previously used in the prevention of hepatitis A infections. The long-term goal would be to provide a completely protective vaccine so that troops could be immunized before deployment. These goals seem appropriate and important. Even with the best of drug therapy, resistance of malaria organisms arises rapidly. We were shown evidence that resistance to current drug therapy occurs after only a few years of widespread use of malaria chemotherapy. Perhaps of equal importance was the revelation that, even during the Vietnam era, when most of the current drugs were available for protection of troops, there was still a significant occurrence of malaria because troops either failed to use personal protective techniques such as bednets and/or had inadequate prophylaxis with drugs that were available. This is likely to be a continuing problem. Only a totally protective vaccine could obviate these serious problems.

2. APPROPRIATE TECHNOLOGY -

The review revealed that the most modern molecular and immuno-biological technologies are currently being used. It was emphasized that there currently is no vaccine available for an organism as complicated as a parasite. Despite this, the new technologies have allowed for an evaluation of the pathogenic events in infection and immunity which seem to provide an understanding which will lead to development of immune protection in malaria. All the appropriate new technologies are being used;

however, it was also quite clear that the research personnel available for new molecular approaches were quite limited, and, therefore, the rapid research using these new techniques is relatively fragile. This could only be corrected by the allocation of additional funds and/or positions to support new technologies.

3. **MEASURABLE PROGRESS** -
The finding that radiated sporozoites vaccination completely protected humans is an observation that is almost 20 years old. It was very important that these observations were recently reproduced. In the University of Maryland study, it has been reconfirmed that radiated sporozoites will produce complete protection against malaria in humans. This gives additional impetus to the search for new antigens which would be the targets of future vaccines. There has been excellent progress in this regard. The identification of this CTL as the main protective mechanism in sporozoite immunization should allow the targeting of resources on this important area. Much of the previous work had dealt with protection provided by antibodies and the humoral immune system.

4. **UNNECESSARY DUPLICATION** -
While there were suggestions that better cooperation and communication between the Army and Navy programs would be beneficial, there was little objective evidence of unnecessary duplication. In only one area was such duplication specifically identified. This related to the development of T cell immunity laboratories in the Army program which duplicated those laboratories already available in the Navy program. Presumably, this was done because of the recent findings which specified that T cell immunity is the important component of the immune response in sporozoite vaccination. Perhaps the utilization of this tissue culture technology could be improved if the T cell laboratories were coalesced into a single laboratory operation. Coordination of vaccine testing must also be emphasized.

5. **PROGRAM DEFICIENCIES** -
There is little doubt that this program is a world-class venture. The vaccine development programs of DOD are recognized internationally as the major definitive program for the malaria problem. The DOD vaccine program, therefore, is an extremely important one which deserves its position as the number one recipient of DOD funds in the area of infectious diseases. However, the number of investigators involved is a precious few. Both in the Army and the Navy, many of the areas of expertise are only "one deep," and a program as important as this one should have some depth. It is somewhat like playing in a professional basketball league with only five players and no one on the bench. This needs to be corrected in one of two ways. Either additional personnel need to be assigned to provide the depth of research capacity to assure its long-term success; or, alternatively, funds should be provided to provide for major contracts for the

development of the essential components of the vaccine program, for example, a contract for the production of the sporozoites, a contract for an animal model which can be used for routine testing of vaccine antigens, or a contract for the production of experimental vaccines that could be used at the initial stages (research level) to identify the most active antigens relative to CTL T8 lymphocyte activity.

The contract program on the drug development aspect of the DOD malaria program is commendable. It allows drug development to proceed with a relatively minimal in-house capability. Some thought should be given to providing a similar level of contract support to vaccine development in order to assure timely progress in malaria vaccine development.

6. **APPROPRIATE INTERACTION -**

A. **Within Institutions:** It appears that interaction within the Army and within the Navy is satisfactory, although distribution of funding—both within the Army and Navy—appears to be misunderstood (or not understood) by the laboratories directly involved in vaccine development. The distribution of overhead, the availability of direct funds, and the overall prioritization of infectious-disease funding was not clear.

B. **Between Laboratories:** It was suggested that there was a lack of communication and understanding of activities between laboratories. It was clear that Dr. Sadof of the Army laboratory has the responsibility for a very large number of vaccines, with approximately 15 to 20 percent of his effort involved in malaria vaccine development. He indicated that he would like to see the Navy lab serve as a basic science laboratory providing products to his laboratory that he could use for the clinical development of vaccines. On the other hand, a concern was voiced by Dr. Hoffman of the Navy laboratories that, in order for them to maintain support of the Navy, they must themselves be involved through the Navy overseas laboratories in major aspects of clinical evaluation. This discussion made it clear that more coordination was required.

It was suggested that one individual should be made responsible for the entire DOD malaria vaccine program. Such an individual would be responsible for making decisions regarding both basic and clinically applied vaccine development research to best utilize the resources available both within the Army and the Navy. This suggestion seems reasonable and would obviate most of the communication problems between laboratories. It was also suggested that the individual responsible for the malaria vaccine program should have full-time commitment to the malaria vaccine development program.

C. **Other Federal Agencies:** There appeared to be good communication with the parasitic diseases laboratories of the National Institutes of Health. The participation in this review by the Director of the Malaria Program for the Agency for International Development suggested an excellent communication with AID.

D. **With Academia and Industry:** There appeared to be good interaction between individual investigators both in the Army and the Navy and their academic colleagues who are participating in malaria research. There also appeared to be excellent interaction with industry. The maximization of the potential vaccine development program through the cooperation of industry is commendable. Such interactions perhaps could be made even more effective if the DOD would consider development of a contract support program, such as is currently available for the malaria chemotherapy program, in order to provide for essential components necessary for development of vaccines for malaria.

7. **RESOURCES -**

A. **Adequate Funding:** It is clear that the funding currently available is minimal for the work outlined. If this program is to be sustained and if a malaria vaccine is contemplated within the next 6 years, then funding will have to be maintained or increased. It was reported repeatedly that funding in the infectious disease area would be going down. The budgets presented showed a decrease in overall funding in the next year or two. It was suggested by the reviewers that the infectious disease program needed to evaluate all of its program objectives and place malaria in the appropriate category of military interest to assure that it receives the funding necessary to retain its current scientific assets and to expand the program sufficiently so that both the vaccine and the chemotherapy area make adequate progress so that we would not be "caught short" in the event of another conflict which might occur in a more malaria-prone area of the world.

B. **Adequate Staffing:** Current staffing is minimal but adequate. There is no doubt that the loss of one or two key people would seriously impair this program. Greater depth of staffing is encouraged, or contracts for more routine development of essential components of the vaccine program need to be developed. There is little doubt that larger staff assignment would lead to a more rapid progress and bring the likelihood for vaccine development into a shorter time cycle. In other words, investment of both funds and staff in this area would certainly shorten the time necessary to develop a protective vaccine for American troops.

8. **OTHER COMMENTS -**
All in all, the DOD should be proud of its rather remarkable efforts in the development of a malaria vaccine. However, we should recognize that until a malaria vaccine is available, we have not adequately solved the problem of malaria in American troops. Even the development of new chemotherapeutic agents would not likely provide us with any greater protection than we were able to achieve in the Vietnam war. This level of protection in American troops is not adequate. Therefore, we should concentrate our efforts on a combined approach of chemotherapy development in vaccine development as a short-term solution and pursue a long-term vaccines program for complete malaria protection.

II. DRUG DEVELOPMENT IN THE MALARIA PROGRAM

1. **CLEAR RESEARCH GOALS -**
The goals for drug development have been relatively clear over the entire history of the program, which now extends to almost 25 years. Basically, the goals are to evaluate currently available drugs to see whether new formulations can circumvent the resistance which so quickly forms to currently available therapies. Second, the evaluation of biological mechanisms within the organism is being used in an attempt to provide information for development of new drug therapies. And third, the utilization of "native" drug therapies from various parts of the world, including China, is being pursued to see whether such drugs can be modified so as to provide malaria protection without the toxicity which has been recently associated with such drugs.

2. **APPROPRIATE TECHNOLOGY -**
On the one hand, the technology seems to be appropriate with very sophisticated capability for analysis of the morphometric aspects of receptor binding and drug configurations. On the other hand, much of the data presented would seem to be based on a trial and error approach. Often the configuration of drugs and their interaction with receptors did not seem to be predictable. This requires, therefore, an extensive system for evaluation of putative drug formulations in order to find those that might be the most active. We were told that most of the drug development had occurred within three families of drugs which were derivatives of prior known active pharmacological agents.

3. **MEASURABLE PROGRESS -**
Progress is easily measurable within the malaria drug development area, as products have already been identified which, at least initially, were effective in control, prevention, or treatment of malaria. This leads to product development, which not only has been useful for the DOD personnel but also has resulted in widespread international use. Unfortunately, it was reported that these drugs rapidly lose their effectiveness, as the malaria organism is very adept at protecting itself from such therapies. It appears that current attempts to reverse malaria resistance may offer some hope for re-activating the potential usefulness of

drugs which had previously been effective in the chemotherapy of malaria.

4. **UNNECESSARY DUPLICATION** -
The only unnecessary duplication that was presented was the evaluation of some drugs which are already available on the international market commercially and were still being evaluated within the chemotherapy program of DOD because they had not yet achieved FDA approval. It might be possible to reduce the overall cost of the program if the producers of such drugs could be encouraged to provide the information necessary for FDA approval of drug usage without the requirement for DOD to produce the clinical evaluations necessary for such approval.

5. **PROGRAM DEFICIENCIES** -
The scientists in this program reported that lack of animal models is a potential area of deficiency in the program. Animal models of malaria were said to be important but not to be totally predictive of eventual effects in man. Expansion of primate modeling was discussed; however, the availability of only a chimpanzee model and/or the Aotus monkey model provided a costly and not necessarily full evaluation of putative drugs. It is important, however, to note that in the evaluation of drugs which had been derived from Chinese native medication for treatment of malaria, it was possible to show serious neurological effects in an animal model system, thus providing insight into toxicity and allowing the testing of humans to proceed with a better knowledge of the potential problems involved.

6. **APPROPRIATE INTERACTIONS** -
   A. **Within the Institution:** The program exists within one section of the Army drug development program and would seem to have adequate and regular communication and interactions between the members of that program.

   B. **Between Labs:** As far as we could tell, there was only one laboratory involved.

   C. **Other Federal Agencies:** The drug development program appears to interact and communicate well with both the NIH and the AID.

   D. **With Academia and Industry:** Several academic centers have provided drugs for evaluation by the Army. The level of scientific interaction with academia seems to be declining as funds available for support of research contracts are diminishing. Contacts with industry would seem to be working very satisfactorily.

7. **RESOURCES** -

   A. **Adequate Funding:** While there is no anticipated major overall reduction in funding, it was apparent that more

rapid progress could be made if additional funding were available. The ability to fund additional support contracts should facilitate progress. In this regard, it should be pointed out that this is one of the major threats facing our troops as they are deployed around the world. Both short-term immediate protection by the development of new chemotherapeutics and longer-term development of vaccines to completely protect the troops must receive a very high priority for funding. At the very least, DOD should make every attempt not to reduce the current funding levels. These programs should not be allowed to dwindle to a level where their progress begins to disintegrate.

B. **Adequate Staffing:** Because so much of the work is done on contract, it was not easy to evaluate whether or not the DOD staffing was adequate to monitor these contracts.

8. **OTHER COMMENTS** –

The world has looked on the drug development program in malaria sponsored by the DOD as an internationally outstanding important effort. There is little doubt that malaria still exists as the major threat not only to the military but also to populations widespread throughout the world. The program in chemotherapy development must be maintained if we are to keep up with the recurring problem of malaria resistance to current drug therapies. While this work may appear to be science on a treadmill, the action of the treadmill must be maintained if we are to provide near-term control and prevent the major complications of malaria in our troops.

DOD Malaria Research Review

May 20-21, 1992

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